

ANNUAL REVIEW OF BIOCHEMISTRY

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PREFACE

These introductory paragraphs are being written on the ninth day of April. The past twenty-four hours have been eventful, tragic, and grimly foreboding. It is almost impossible to write calmly, objectively, and with that sense of detachment that all of us cherish. The pursuit of learning and all scholarly and creative activities are thrown into the gravest peril by these ever constant emotional crises, wanton barbarities, and sufferings of the present time. Without the establishment of peace and the construction of a rational social order in which the elementary principles of liberty, justice, and human kindness are recognized as fundamental, there may be no escape from an almost total eclipse of learning, let alone the gravest of suffering to all. There is nothing of value that has thrived on fear, and what good end has ever been attained by violence?

That men thousands of miles from the areas of actual conflict should feel so keenly the grimness and tragedy of the present is an inevitable consequence of the extraordinary bond of understanding and good will that unites scholars everywhere and brings them in closest sympathy with all mankind.

It may well be surmised that this present volume of the *Review* has been prepared amid unusual difficulties that need hardly be mentioned in any detail. We feel that nothing can be said that would express adequately the gratitude that is ours for the conscientious efforts that have been expended by our many collaborators in writing the various reviews. We trust that we may mention specifically, however, the assistance that has been given so unstintingly by our colleagues abroad. The unexpected obligations and trying duties imposed by the stress of war rendered difficult the sacrifice of time for writing; journals of original publication were not always at hand; delays in the delivery of mail were endless. The reader will appreciate, without further elaboration, the almost obvious difficulties that had to be overcome.

It is a matter of profound regret to us that the review on muscle expected from Professor Parnas of Lwow, who was among the first contributors to the *Review*, did not arrive. If received within the next few weeks other provisions will be made for its publication.

We cannot conclude without expressing most sincerely our thanks to those who have helped us greatly in a variety of ways, as yet unmentioned. There are those who have been good enough to convey to

us suggestions, always received with welcome, in respect to authorship, topics, the respective merit of individual reviews, errors in content, and matters of editorial policy. Others continue to send to us or to the authors of the various reviews reprints of published work, a service of considerable value if the journal of original publication is relatively obscure or inaccessible. Finally, we wish to express our very great appreciation of much editorial help that was given by Professor H. S. Loring, and of splendid co-operation by the editorial assistants, and the Stanford University Press.

J. M. L.
C. L. A.
D. R. H.
J. H. C. S.
C. L. A. S.

ERRATA

Volume VIII, page ix, line 17: *for* Phosphorous, *read* Phosphorus.

Page 47, line 8: *for* formaline, *read* formalin.

Page 69, line 8: *for* Ohlson, *read* Ohlsson.

Page 79, reference 101: *for* Ohlson, *read* Ohlsson.

Page 179, line 1: *for* Burgers, *read* Burgess.

Line 10 from bottom: *for* Dyer, H. W., *read* Dyer, H. M.

Page 287, line 12; *for* phosporus, *read* phosphorus.

Page 295, reference 6: *for* Maynard, L. C., *read* Maynard, L. A.

Page 395, line 7 from bottom: *for* Pulfric, *read* Pulfrich.

Page 408, reference 29: *for* Lu, G. C., *read* Lu, G. D.

Page 412, reference 79: *for* Pillemer, I., *read* Pillemer, L.

Page 429, line 20: *for* ineffective, *read* effective.

Page 435, line 6 from bottom: *for* hours, *read* minutes.

Page 451, line 2: *for* functions, *read* junctions.

Page 500, references 17 and 18: *for* Franck, I., *read* Franck, J.

Page 577, reference 79: *for* McClure, F. G., *read* McClure, F. J.

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BIOLOGICAL OXIDATIONS AND REDUCTIONS

BY KURT G. STERN

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Work in the field of biological oxidation during the year 1939 has made notable advances at many points along a far-flung front. However, there have also been reverses and impasses. In the preparation of this survey full advantage has been taken of the privilege extended by the Editorial Committee to examine critically only a selected number of contributions and to concentrate on those phases in which, in the judgment of the reviewer, the most significant developments have taken place. In order to provide the background required for an appraisal of the events of 1939, it has been found necessary to draw upon a number of earlier publications.

RESPIRATORY FERMENT

Cytochrome- a_3 and the respiratory ferment.—Probably the most widely discussed contribution to the subject during the past year has been the paper by Keilin & Hartree (48), on the discovery of a new cytochrome component (a_3) and its relationship to Warburg's oxygen-transmitting enzyme of respiration (for short, respiratory ferment). The essence of the paper is contained in the following diagram (Fig. 1), as given by the authors.

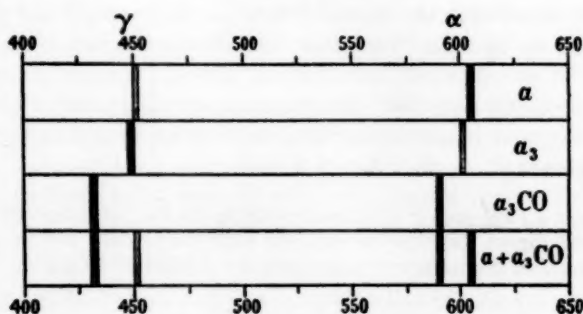


FIG. 1.—Diagram showing the relationship between the bands of cytochrome components a and a_3 and the effect of carbon monoxide on a_3

It is concluded that the absorption bands heretofore ascribed to the cytochrome-*a* component are actually the fused bands of *a* and *a*₃. The proof is largely seen in the observation that treatment with carbon monoxide leads to a splitting of the cytochrome-*a* absorption bands: a new band appears at 590 mμ and a large fraction of the γ-band at 448 mμ is shifted to 432 mμ, thereby causing a large increase in intensity of the γ-band of cytochrome-*b* which is situated in the same region. That portion of the original cytochrome-*a* bands which is not shifted by carbon monoxide is assigned to the "true" cytochrome-*a* which is not autoxidizable and does not combine with carbon monoxide, whereas the two new bands are attributed to the hitherto unknown *a*₃ component which is autoxidizable and forms, when in the ferrous form, reversible compounds with carbon monoxide and, when in the ferric state, with potassium cyanide, hydrogen sulfide, sodium azide, and hydroxylamine.

The *a*₃ compound is identified with cytochrome oxidase as well as with the oxygen-transmitting enzyme of Warburg. It is admitted, however, that this theory has a few weak points. Foremost among these is the inability to demonstrate a direct or indirect reduction of oxidized cytochrome-*a*₃ by reduced cytochrome-*c*. Another is the stability of the carbon monoxide complex of cytochrome-*a*₃ to strong illumination although Warburg has shown that the carbon monoxide inhibition of respiration may be relieved by light. Last but not least the authors state that on shaking a heart muscle oxidase preparation with air in the presence of succinic acid and carbon monoxide the cytochrome components *a*, *b*, and *c* undergo oxidation while the spectrum of *a*₃·CO remains still clearly visible. This experiment seems to indicate that the components *a*, *b*, and *c* could not possibly be oxidized by *a*₃ since the latter remained combined with carbon monoxide.

In a preceding note (44) incorporating much of the evidence reported in the later paper the authors were so impressed by the objections mentioned above that they felt that

the component *a*₃, although it may be responsible for the photochemical absorption spectrum, cannot therefore be identified with the oxidase, and the problem as to the nature of the enzyme remains open for further investigation.

They discuss the possibility that the respiratory enzyme may be a hematin-protein compound similar to cytochrome-*a* but, owing to its possible low concentration, spectroscopically invisible even in strong

enzyme preparations; or it may be an iron-protein compound devoid of porphyrin; finally, it may be a copper-protein compound similar to polyphenol oxidase. The last possibility is favored in the preliminary publication.¹ Upon reconsideration, however, the objections against an identification of cytochrome- a_3 with the respiratory ferment are found to be "only apparent." The fact is stressed that the experimental conditions during the spectroscopic observations are very different from those obtaining during catalytic, i.e., kinetic, experiments. In discussing the relationship between the a and a_3 components, the authors state that the α -band of a_3 is weak compared with that of a , while, on the contrary, the γ -band of a_3 is much stronger than that of a . If the relationship is actually that described and sketched by the authors, it can hardly be reconciled with the further statement that the a and a_3 components are both heme-protein complexes with an identical heme nucleus and that both occur in comparable concentrations in various oxidase preparations. It is a general rule that all compounds that contain an intact porphyrin nucleus have γ - or Soret bands in the blue-violet region; these have a much higher extinction than any of the α - or β -bands situated in the green, yellow or red.² According to Keilin & Hartree, however, this relationship is reversed in the instance of the cytochrome- a component. This, on the basis of a large body of data, would not only militate against a classification of this component as a porphyrin derivative but it would also rule out any close chemical relationship between cytochrome- a and $-a_3$. There exists, however, the possibility that the relative intensities of the various absorption bands were misjudged due to the low visual acuity of the human eye in the blue-violet region and that a subsequent study by an objective spectrophotometric method may yet reveal a "natural" order in the extinction ratios of the various absorption bands concerned. Even if one should accept Keilin & Hartree's postulation of the existence of the new cytochrome- a_3 component, its identity with the oxygen-transmitting enzyme of Warburg cannot be considered as established at this time. Several facts which have been mentioned seem to militate strongly against this view and their reconciliation with the new theory can only be attempted by further experimentation.

¹ See also the recent experiments of King and his associates (31) who find that respiration and photosynthesis in *Chlorella* are inhibited by organic compounds which form complexes with copper.

² Cf. Holden & Lemberg (38).

The paper gains in interest when viewed from the historical perspective, in that only a few years ago Keilin (42) contested the claim of Warburg and his associates (cf. 111), that the absorption bands of the respiratory ferment may be directly observed in certain microorganisms, on the ground that these bands belong to derivatives of the labile and easily modified cytochrome-*a* component (cytochrome-*a*₁ and -*a*₂) which have acquired the properties of artificial hemochromogens in reacting with molecular oxygen and carbon monoxide. At that time Warburg (114) objected to the classification of hemin compounds of very different properties under a common heading, viz., cytochrome. An analysis of the reports of Warburg and his co-workers (113) shows that their arguments for an identification of the "cytochrome-*a*₁" component with the oxygen-transmitting enzyme were certainly as convincing, if not more so, than those now advanced by Keilin & Hartree in favor of their latest hypothesis: the α -band of the carbon monoxide compound of "cytochrome-*a*₁" is situated at the position predicted by the photochemical experiments of Kubowitz & Haas (55); "*a*₁" reacts with molecular oxygen and hydrogen cyanide, and the combination of this hemin compound with cyanide blocks the reoxidation of the other nonautooxidizable cytochrome components as would be expected from a catalyst which fulfills the function of a cytochrome oxidase or of Warburg's respiratory ferment. A decision on the question of the identity of the "respiratory" absorption band in the red region in *Azotobacter* (76) with the α -band of the respiratory enzyme, which in this case would be a chlorophyll hemin or biliviolin rather than a pheohemin derivative, will have to await the charting of the photochemical absorption spectrum of this microorganism.

On the isolation of cytochrome oxidase.—Warburg is frequently identified with the statement that the respiratory ferment is so intimately linked up with the architecture of intact cells that any attempt at purification and isolation of the enzyme is doomed to failure. Actually, Warburg demonstrated as far back as 1913 that the mechanical destruction of liver tissue yields suspensions of minute granules which show an intense respiration. The postulate of the structure-bound nature of cell respiration referred to such protoplasmic, presumably preformed, granules rather than to intact cells. By employing a modified procedure of Battelli & L. Stern for the preparation of "indophenol oxidase" Keilin & Hartree (46) have recently obtained preparations from pig's heart muscle which contained an active cytochrome

oxidase, cytochromes-*a* and -*b*, and succinic dehydrogenase, as well as other components. For a strong catalytic activity towards *p*-phenylenediamine and, even more so, towards hydroquinone the addition of cytochrome-*c* was found necessary. The authors consider their preparations to represent finely divided suspensions of muscle tissue containing the cytochrome oxidase and other components in an insoluble state. A physicochemical study of such materials in the ultracentrifuge and in the electrophoresis apparatus (cf. 92) has revealed a number of properties also observed in certain macromolecular materials, e.g., in purified fractions from Rous chicken sarcomata and from the tissues of leukemic fowls, which have been linked to the activity of animal viruses. A similar material has recently been isolated from normal chick embryos by Claude. All of these materials seem to consist of nearly symmetrical particles³ of a "molecular weight" of the order of hundreds of millions. Besides protein they contain lipid material, nucleic acid, hemin, in short most constituents of protoplasm. According to studies in the analytical ultracentrifuge their degree of homogeneity is low but probably not lower than that of tobacco mosaic virus protein after damage by contact with strong salts and recrystallization.

The isolation of a water-soluble cytochrome-*c* oxidase from both brewers' and bakers' yeast has recently been announced by Hogness and his co-workers (6). The enzyme is precipitable by ammonium sulfate and alcohol, is thermolabile, and cannot be sedimented from its water-clear solution by centrifuging for one hour in a field of 35,000 g. The activity of the enzyme is determined by measuring

³ Whether essentially monodisperse suspensions of such particles are called mechanical dispersions or true solutions is largely a matter of definition. Just as most proteins form colloidal solutions because the individual molecules reach the order of magnitude of a colloidal micelle, it is conceivable that a further increase in size may bring single molecules or well-defined molecular complexes into the realm of mechanical suspensions. It is quite possible that further work may succeed in resolving the large "functional" aggregates into their individual components which may still exhibit their catalytic activity in suitable test systems [cf. Hopkins *et al.* (39)]. But it is certain that such a subdivision will destroy the degree of organization which enables the intact particles to display their characteristic biological activity either as complete oxidase systems or as virus particles. Whether these complexes contain a common bearer protein studded with a number of prosthetic or active groups or whether they are systems of individual complete enzyme molecules cemented together in an orderly fashion, remains to be determined.

spectrophotometrically the rate of oxidation of reduced cytochrome-*c* by dissolved oxygen in the presence of small amounts of the enzyme preparation. The test reaction is inhibited by potassium cyanide, carbon monoxide, and, strangely enough, also by small amounts of catalase. It has not yet been possible to demonstrate any enzymatic catalysis in the oxidation of *p*-phenylenediamine or hydroquinone by molecular oxygen in the presence of cytochrome-*c*, although such an activity is generally attributed to cytochrome oxidase. Furthermore, only minute amounts of the enzyme have thus far been extracted from large quantities of yeast and only special yeast strains have been found to yield active preparations. It is difficult, at the present time, to assess the quantitative significance of this enzyme for the main respiration of the cells.

The oxidation of cytochrome-*c* in vitro by a number of nonenzymatic agents, e.g., hydrogen peroxide, copper salts, etc., has been observed by Keilin (41). Hogness and his co-workers have demonstrated that metal ions such as iron, copper, and manganese, cannot be responsible for the catalytic activity of their enzyme preparation. But the inhibitory effect of catalase seems to point to hydrogen peroxide as the agent actually oxidizing the ferrocytochrome-*c* in the test system employed. This, in turn, makes it necessary to explain the mode of formation of the hypothetical peroxide. In this connection it is also noteworthy that, according to Keilin & Hartree (43), cytochrome-*c* is biologically oxidized only by indophenol oxidase (cytochrome oxidase) but not by catechol oxidase. However, polyphenol oxidase is able, in the presence of very small amounts of catechol, to oxidize many substances, such as reduced coenzyme, hemoglobin or ascorbic acid (47). It does not seem impossible, in view of the high oxidation potential of the intermediary orthoquinone formed in the reaction from catechol, that ferrocytochrome-*c* is also oxidized under these conditions. The water-soluble *Lactarius* oxidase will oxidize cytochrome-*c* slowly even in the absence of a phenolic substrate (46).

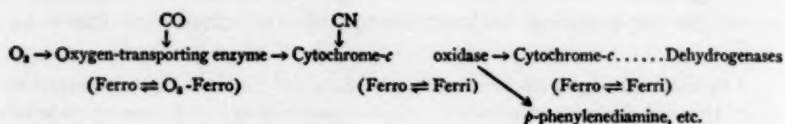
The claim of Yamaguchi, Tamiya & Shibata (cf. 46) to have obtained clear aqueous solutions of indophenol oxidase (cytochrome oxidase) from yeast, heart muscle, and mushrooms has been contested by Keilin & Hartree (46).

On the identity of cytochrome oxidase and oxygen-transmitting enzyme.—While the identity of these two biocatalysts is generally accepted by European and American workers, the Japanese school of Shibata, Tamiya and their associates has for years maintained that

indophenol oxidase (cytochrome oxidase in the new nomenclature) and the respiratory enzyme are different compounds.⁴ The earlier claim of these workers (cf. 87) that cytochrome itself is capable of reversible oxygenation without change of the valency of the iron,

⁴ Previous attempts by workers from the same school to demonstrate the dual nature of the point of attack of carbon monoxide and hydrogen cyanide on the so-called Warburg-Keilin respiratory system had been criticized on the ground that those observations had been made with cell-free extracts which would hardly permit an accurate characterization of a system which is exerting its biological function while intimately bound to the structure of the cell. For this reason the authors chose living acetic acid bacteria and yeast cells for their new experiments (104). The equation of Warburg for the distribution of the respiratory enzyme between oxygen and carbon monoxide is based on the assumption that in the presence of the two gases the entire amount of enzyme is saturated either with carbon monoxide or oxygen. One of the constants contained in that expression, defined as the rate constant of the "consumption" of the oxidized form of the enzyme (FeO_2) by the respiration, is apparently based on the further assumption that upon saturating the "surface" of the cell with fuel substances this breakdown of FeO_2 proceeds with a definite velocity which is independent of the nature of the substrate. The authors contend that the actual value of this rate constant must be affected by the nature of the fuel substance as well as by all factors which interfere with any component of the chain of catalysts which acts like a reductant on the respiratory ferment oxygen compound. The activity of this reducing chain was varied by changing the type of fuel as well as by the narcotic ethylurethane. By measuring the respiratory intensity in the presence and absence of carbon monoxide the effect of such changes on the value of the distribution constant of the enzyme between carbon monoxide and oxygen was determined. It was found that the greater the activity of the "reducing system," the more pronounced was the extent of carbon monoxide inhibition of respiration and the smaller the distribution constant k . When the concentration of the enzyme was kept constant by using the same organisms and when the activity of the reducing chain was varied the equation $Q_{\text{max},1}/Q_{\text{max},2} = k_2/k_1$ was found to fit the results. Here, k designates the distribution coefficients and Q_{max} , the maximal respiration of the cells upon saturation with oxygen and in the absence of carbon monoxide. This latter value was proportional to the activity of the "reducing chain." It could be shown, furthermore, that the same relation held if the respiration were partly inhibited by HCN and if the effect of CO/O_2 gas mixtures were tested on the remainder of the respiration. The authors explain this observation by the hypothesis that cyanide, in analogy with urethane, does not react with the oxygen-transmitting enzyme proper but with a component of the "reducing chain." The chain link actually concerned is identified with cytochrome oxidase (indophenol oxidase) which the authors state to be cyanide-sensitive. According to the scheme reproduced above this enzyme accepts molecular oxygen from the oxygenated respiratory ferment of Warburg which is stated to be carbon monoxide-sensitive but practically cyanide-resistant.

seems to have been abandoned. Their present concept culminates in the following scheme (104):



One of the most interesting points in this contribution is the view that the combination of the respiratory enzyme with oxygen does not involve a valency change of the iron from the ferrous to the ferric state but that it is analogous to oxyhemoglobin where molecular oxygen is "loosely" bound to the ferrous form of the pigment. The authors point out that the best spectroscopic models yet found by Warburg for his enzyme are compounds of hemoglobin character, such as chlorocruorin and the synthetic hybrid compound *Spirographis* hemoglobin. Warburg himself has envisaged the possibility of the formation of a complex between the respiratory ferment in its ferrous form and molecular oxygen. Such a complex, however, is thought to represent an intermediate of greater or lesser stability which is eventually transformed into the ferric compound by intramolecular oxidation. The ferric form of the enzyme is considered by Warburg to be the true oxidizing agent in respiration (111). However, there exists no direct experimental evidence for this valency shift in the respiratory ferment during its catalytic function. The scheme proposed by Tamiya & Kubo has some attractive features and deserves further experimental scrutiny. It will be noted that the oxidation of the unphysiological substrate *p*-phenylenediamine is pictured in their scheme as being independent of the intervention of cytochrome-*c* which is in contrast to the views and experiments of Keilin & Hartree (46). A final decision on this point will only become possible with the preparation of oxidase preparations which will not attack this substrate at all in the absence of cytochrome-*c*.

CYTOCHROME

In last year's review (26) some recent work by Theorell (106) on the constitution of cytochrome-*c* was reported.⁵

⁵ In continuation of the earlier experiments of Zeile and his co-workers (119) on the structure of cytochrome-*c*, Theorell, with the aid of pure, or

Upon reinvestigating the problem with particular attention to the events occurring during the acid hydrolysis of cytochrome-*c* Theorell (108) has now revised his earlier conclusions. He was able to show that proto- or hematoporphyrin (but not mesoporphyrin), upon heating with cysteine in hydrochloric acid, will yield products which correspond in every respect to the "porphyrin-*c*" previously isolated from acid cytochrome-*c* hydrolysates. He considers porphyrin-*c* to be very probably an artefact which arises in the course of the cytochrome hydrolysis by a secondary reaction between the porphyrin and free cysteine split out from the protein. The product thus formed probably represents a mixture of α - and β -substituted porphyrins. While these findings would seem to rule out the existence of a thioether or other sulfur-containing bridge in native cytochrome-*c*, they do not affect the basic concept of Theorell that here, in contrast to hemoglobin, the heme residue is linked in two different ways to the protein, viz., by stable main valency bonds between the protein and one or two vinyl side chains of the porphyrin, as well as by the usual coordinate bonds between the central iron atom and some hemochromogen-forming groupings in the protein component. The main valency link could be formed with the aid of nitrogen or oxygen atoms.⁶ Zeile, too, has

nearly pure, cytochrome-*c* from heart muscle, had arrived at the conclusion that the prosthetic hemin group is linked to the bearer protein through a thioether bridge between the vinyl side chains of the porphyrin in the 2- and 4- positions and amino acid residues constituting a part of the protein moiety. This formulation was largely based on the study of a product obtained upon hydrolysis of cytochrome-*c* by strong hydrochloric acid at 100°. The analysis of this product showed it to contain porphyrin, sulfur and amino acid residues in definite proportions. This compound which displayed the same general properties as a porphyrin obtained previously by different procedures from cytochrome-*c* by Hill & Keilin as well as Zeile was considered by Theorell to represent the native cytochrome-*c* porphyrin (for short, porphyrin-*c*). The breakdown of the iron-complex salt of this compound with hydrobromic and acetic acids yielded mainly hematoporphyrin and *l*-cystine (107), which suggested that cytochrome-*c* arises through the reaction of the vinyl groups of the porphyrin with two cysteine residues of the protein to form the bridge $\text{COOH}\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2\cdot\text{S}\cdot\text{CH}\cdot\text{CH}_3$.

⁶ It will be recalled that Zeile, on the basis of his synthetic experiments (cf. 119), suggested that one or two of the vinyl side chains in cytochrome-*c* may be combined with a tertiary nitrogen base. The observation of Theorell that the condensation of porphyrin with cysteine through the sulfur atom also yields a product with the general properties of the natural cytochrome-*c* porphyrin, demonstrates that a nitrogen base is not the only possible substituent. Furthermore, the examination of the cytochrome hydrolysates after removal of the

continued his earlier experiments on the structure of cytochrome-*c* and has now published an extensive paper on the subject (120). Independently and simultaneously with Theorell he had arrived at the conclusion that sulfur atoms form the bridging link from the heme residue to the protein part of the molecule. Upon the publication of Theorell's revision of his earlier claims, Zeile reinvestigated the question and obtained further evidence that the hypothesis involving sulfur bridges has a high degree of probability. Thus it can be shown that the acid hydrolysis of cytochrome yields the porphyrin-cysteine compound described by Theorell even under conditions where the possibility of a resynthesis from primary products is very remote as demonstrated by synthetic control experiments. Upon introducing iron into the porphyrin-cysteine compound a typical hemochromogen band at 550 mμ appears although no extraneous nitrogenous substance is present. Zeile concludes that the hemochromogen formation in this instance is of an intramolecular type, i.e., hemochromogen formation takes place between the nitrogen atoms of the amino acid side chain and the metal atom. It is inferred that in cytochrome-*c* there exists no separate hemochromogen-forming nitrogenous component which is distinct from the protein but that the heme forms a hemochromogen with suitably situated nitrogen atoms of the protein, perhaps with those in the cysteine side chain. Upon warming ferrocyclochrome with hydrosulfite and hydrochloric acid, the iron is split out readily (in contrast to ferricytochrome). By this procedure no separate low-molecular nitrogenous component is released. The lack of autoxidizability of cytochrome-*c* is explained by the spatial fixation of the hemochromogen-forming nitrogen atoms in the molecule, in contrast to the artificial hemochromogens where dissociation equilibria exist between the hemochromogen and its components.

PASTEUR REACTION AND PASTEUR ENZYME

When Louis Pasteur, in 1861, published his study on the *Influence of Oxygen on the Development of Yeast and on Alcoholic Fer-*

porphyrin-cysteine compound failed to show the presence of a tertiary nitrogen base (108). The possibility that neither sulfur nor nitrogen but oxygen may form the bridging link in cytochrome-*c* is discussed by Theorell. The link could conceivably be formed by the reaction of an hydroxyl group with a vinyl side chain of the porphyrin. In this connection it is of interest that pure cytochrome-*c* has been found to contain carbohydrate in sufficient amounts (105).

mentation and then further elaborated on this problem in 1876, in his classical treatise, *Études sur la Bière*, he not only presented the world with a fundamental discovery but he also initiated a controversy which today continues unabated. Pasteur showed that in contrast to the usual or aerobic type of cell metabolism where the amount of foodstuff used is not much greater than the mass of cell material synthesized with its aid, the weight of sugar destroyed in alcoholic yeast fermentation is inordinately larger than the weight of yeast substance formed. With increasing aeration of the culture medium the ratio of sugar destroyed to yeast substance formed was found to decrease. Although Pasteur clearly appreciated the improvement in energetic economy upon transition from an anaerobic type of metabolism, i.e., alcoholic fermentation, to the aerobic type, i.e., respiration, he did not furnish clear-cut evidence that the rate of carbohydrate breakdown in a given time is decreased by the admission of air. His experiments were complicated by their long duration and by the fact that the yeast not only multiplied during the experiment, thereby varying the amount of "ferment" available, but that the "vitality" or the "power of the ferment" also changed in the course of the experiment. This rendered the interpretation of Pasteur's experiments so ambiguous that Schützenberger, proposing a measure of fermentative energy on the basis of the quantity of sugar decomposed by unit-weight of yeast in unit-time, drew from these observations conclusions which were exactly opposite to those deduced by Pasteur. In fact, the essential point in Pasteur's theory, viz., that the rate of sugar decomposition in a given time is less in the presence than in the absence of air, was first clearly established by Meyerhof's experiments with yeast in 1925.

The development of the subject has been traced by Lohmann (65), K. C. Dixon (25), and D. Burk (23).

One of the most controversial points with regard to the phenomenon discovered by Pasteur concerns the question whether the process of respiration or the oxygen gas itself is responsible for the decrease in fermentation upon transition from anaerobic to aerobic conditions. Pasteur himself unquestionably favored the former view. When Meyerhof developed his theory of the oxidative resynthesis of carbohydrate from primarily formed anaerobic fission products, the Pasteur effect, as a curbing of fermentation by respiration, appeared to receive a basis firmly founded on energetic stoichiometry. At about the same time (1926) Warburg, more cautious, defined the Pasteur reaction as the inhibiting effect of respiration on fermentation and as

a quantitative measure he introduced the "Meyerhof Quotient" (decrease in glycolytic fission of carbohydrate caused by oxygen)/(carbohydrate oxidized). This is derived from experimental data and, as such, is independent of the reality of the Meyerhof cycle. In speaking of the "effectiveness" of respiration on fermentation and in ascertaining the extent to which the Pasteur reaction operates in various tissues and under various conditions by the indirect method of determining the value of the Meyerhof quotient, Warburg did not elaborate on the question of mechanism beyond suggesting that the inhibition of the Pasteur reaction by isocyanide is due to interference with the "coupling link between respiration and fermentation." The comparatively brief period since 1926 has not only witnessed an eclipse of the Meyerhof cycle as a general explanation of the Pasteur phenomenon but a rising tide of contributions that tend to disprove the view of a "seesaw" relationship between fermentation and respiration. Only a few of the more significant demonstrations of this kind can be mentioned here: the reversible inhibition of yeast maceration juice fermentation and muscle extract glycolysis by oxidation-reduction systems of a positive potential range (61); the decrease of respiration and constancy of aerobic glycolysis upon lowering the oxygen tension of leucocytes, erythroblasts, and various bacteria (51, 52, 53); and the opposite relationship in the case of various mammalian tissues, such as intestinal mucosa (22) and retina, chorion, mouse sarcoma and liver (56). As a consequence of these and other experiments the concept is rapidly gaining recognition that it is the oxygen tension rather than the respiration which is instrumental in suppressing fermentation in air. The most comprehensive and least vulnerable definition of the Pasteur effect at the present time seems to be the one by K. C. Dixon (25) who considers the Pasteur effect as the action of oxygen in diminishing carbohydrate destruction and in suppressing or decreasing the accumulation of the products of anaerobic metabolism.

If this view be accepted then the question arises whether the oxygen acts by directly and reversibly inhibiting a component of the fermentation enzyme complex or whether there is interposed between oxygen and the fermentation system a catalytic substance capable of reversible oxidation-reduction and of "activating" the oxygen for this function in a manner similar to the activation of oxygen for respiratory purposes by Warburg's respiratory ferment. The fact that the fermentation in cell-free extracts from yeast or muscle is not affected

by molecular oxygen and the restitution of the Pasteur effect by positive oxidation-reduction systems (61) appears to favor strongly the existence, within the cell, of a coupling link between oxygen and the glycolytic enzyme system. The finding of Laser (57), that carbon monoxide will inhibit the Pasteur reaction in mammalian tissues under conditions where the respiration is not affected and that the carbon monoxide inhibition is relieved by light, hardly admits of any other alternative.

Many facts that are now established in respect to the properties of iron-proteids permit us to postulate that such substances may act between oxygen and an oxidizable component of the fermentation system. Under anaerobic conditions the latter component, e.g., a coenzyme of fermentation, would be reduced and reoxidized by intermediary reaction products of the fermentation process (see below). Upon admission of molecular oxygen the iron catalyst, for which the name *Pasteur enzyme* is suggested, would react with the reduced coenzyme and thereby interfere with the normal progress of fermentation. When carbon monoxide is allowed to compete with oxygen for the ferrous form of the Pasteur enzyme, the enzyme iron will distribute itself between the two gases in accordance with its affinities for oxygen and carbon monoxide, and an inhibition of the enzyme will result, depending in magnitude on the CO/O_2 ratio and the distribution coefficient of the enzyme. The reduced fermentation coenzyme will now be reoxidized, at least partly, by a fermentation intermediate and the rate of fermentation will increase accordingly. Upon illuminating the system, the distribution of the iron in the Pasteur enzyme will be shifted in favor of the combination with oxygen and the inhibitory power on fermentation will return, at least partly, for the duration of illumination. All these phenomena are formally quite analogous to those observed by Warburg in the respiration of several microorganisms such as yeast and acetobacter.

Although perhaps the greatest interest in this connection is commanded by tumor tissue which, according to Warburg and in contrast to most normal tissues, exhibits invariably an appreciable aerobic glycolysis both *in vivo* and *in vitro*,⁷ retina tissue lends itself more readily

⁷ The mere demonstration of aerobic glycolysis is inadequate for determining whether the Pasteur effect is operative in a given tissue. In fact, in most tumors examined by Warburg the Meyerhof quotient was found to be within the normal limits, i.e., the Pasteur effect was not impaired. Only in certain tumors

for an enquiry into the nature of the Pasteur enzyme. Warburg has shown that rat retina, at least *in vitro*, has a metabolism somewhat resembling that of tumor tissue, only at a much higher level of intensity; furthermore, the Pasteur reaction in retina is selectively inhibited by carbon monoxide (57) and this inhibition is reversibly relieved by light (110).

The complete absorption spectrum of the Pasteur enzyme in retina is at present being measured by Warburg's photochemical method by J. L. Melnick & D. DuBois (95) in collaboration with the writer. The photochemical efficiency ratios for sixteen different wave lengths of visible light as referred to 436 m μ as the standard wave length have thus far been determined. From the results obtained up to the present it appears that the γ - or Soret absorption band of the Pasteur enzyme in rat retina is situated in the neighborhood of 450 m μ while the α -band has its maximum near 580 m μ . In comparison with the spectrum of the respiratory ferment in yeast or acetobacter (55) the main band of the Pasteur enzyme shows a red shift of approximately 150 Å and the band in the yellow shows a blue shift of about 140 Å. While it is evident that the Pasteur enzyme in retina differs from the respiratory ferment in the same tissue and from that in yeast or acetobacter by its affinity for carbon monoxide and oxygen and from the latter by the position of the absorption bands of the carbon monoxide complex, the general pattern of the Pasteur enzyme spectrum shows it to be a porphyrin-iron proteid. The fact that the α -band is in the yellow region, at about 580 m μ , suggests that the hemin group of the Pasteur enzyme belongs to the class of mixed colored, or pheohemins, which comprises also the prosthetic groups of Warburg's respiratory ferment in yeast and acetobacter, of the worm blood pigment chlorocruorin, and very probably also of certain cytochrome-*a* components. About the nature of the respiratory ferment in retina nothing is known at present.

The function of the Pasteur enzyme is perhaps most readily understood on the basis of one of the explanations of the Pasteur effect

studied by Crabtree and later by Warburg, which were distinguished by a high respiration as well as by aerobic glycolysis, the Meyerhof quotient was low, indicating a disturbance in the Pasteur reaction. One might, of course, adopt the view that the persistence of aerobic glycolysis signifies, under all circumstances, a biochemical lesion.

involving the oxidative removal of some essential intermediate in the glycolytic chain. As intermediates affected in this manner α -glycero-phosphate, pyruvate, acetaldehyde, triosephosphate, etc., have previously been suggested (25). In the recent scheme of Ball (8) the chief emphasis is placed on the rôle of diphosphopyridine nucleotide (cozymase).

According to a diagrammatic representation given by Ball (8) the reaction by which the dihydropyridine nucleotide is reoxidized determines the character of the over-all process: In air the reoxidation is accomplished by the diaphorase (flavoprotein) \rightarrow cytochrome \rightarrow respiratory ferment \rightarrow O_2 chain; under anaerobic conditions the reduced coenzyme reacts with pyruvic acid (in animal tissues) or with acetaldehyde (in yeast) to yield the end products of fermentative metabolism, viz., lactic acid and alcohol respectively. This scheme, which is based on the work of Embden, Meyerhof, Warburg, Euler, Parnas, Barron and others, explains satisfactorily the manner in which oxygen suppresses or diminishes the accumulation of the main products of anaerobic metabolism. On the other hand it fails to account for the decrease of the rate of carbohydrate breakdown in air as well as for the differential effect of certain inhibitors such as carbon monoxide or isocyanide and of varying the oxygen tension on the Pasteur reaction and on respiration. The last-mentioned shortcomings in the scheme may be remedied by insertion of the Pasteur enzyme as the specific oxidant either of dihydrocozymase or of reduced thiamin-pyrophosphate (coenzyme of pyruvic dehydrogenase). There still remains the question as to the cause of the diminished over-all rate of carbohydrate breakdown in air as compared with anaerobiosis. The suggestion (8) that the greater energy yield of total combustion of sugar as contrasted to anaerobic fission causes the cell to slow down or speed up the rate of carbohydrate destruction according to its energy requirements endows the cell with an insight which is almost human.

RESPIRATORY MEDIATORS*

The "unitarian" theory of cellular respiration states that oxygen, after being activated by the respiratory ferment-cytochrome system,

* The writer wishes to acknowledge, with appreciation, the co-operation of Dr. P. P. Cohen in the preparation of this section.

reacts with hydrogen atoms of the metabolites which have been activated or labilized by substrate-specific dehydrogenase systems. The latter process has been shown to consist in the transfer of hydrogen atoms from the substrates to pyridine coenzymes under the influence of catalytically active proteins. It is also established that the actual oxidizing agent in cellular respiration is not molecular oxygen but the ferric iron of the Warburg-Keilin system, and that the sole function of the oxygen consists in the reoxidation of the ferrous iron formed by the reaction with hydrogen. The concept that ferricytochrome reacts directly with the dihydrocoenzymes has proven to be oversimplified. The exploration of the links coupling the iron systems with the dehydrogenases is today the foremost problem in cellular respiration. It is proposed to use the name "respiratory mediators" for these interposed catalysts.

The nature of the respiratory mediators appears to vary with the nature of the substrate, of the coenzymes involved, and with the cell type. It may possibly vary also with the state of metabolic activity, in other words with the demand on the efficiency of cell respiration under varying external conditions. Some of the relationships thus far postulated will be found in Figure 2.

Whereas flavin-adenine-nucleotide in conjunction with specific proteins (diaphorase, coenzyme factor) is now considered to be one of the most important respiratory mediators (see also the section on flavoproteins) the physiological significance of the "old" yellow ferment, which is a flavin phosphoric acid-proteid, is becoming increasingly doubtful. It may, in fact, be the breakdown product of a more complicated flavoprotein, as Warburg suggests.

Flavin-adenine-dinucleotide-proteids, however, do not seem to be the only respiratory mediators of importance in normal cell respiration. Thus Haas (35), working in Hogness' laboratory, is engaged in the isolation of an enzyme from bottom yeast which catalyzes the reaction between ferricytochrome-*c* and dihydrotetraphosphopyridine nucleotide plus activating protein. This factor is different from the new yellow enzyme (flavin-adenine-dinucleotide-proteid) prepared previously from bottom yeast by the same worker (34). The new catalyst appears to be colorless.* Another respiratory mediator has been

* M. Dixon (27) mentions the possibility that this mediator might be identical with a factor which links yeast lactic acid dehydrogenase with cytochrome and which is lost from the preparation upon purification.

found during some recent work on the physicochemical properties of heart muscle oxidase (92). The presence of this principle is required for the aerobic but not the anaerobic oxidation of succinate by the oxidase system (93). This mediator may be removed from the system either by repeated isoelectric precipitation or ultracentrifugal sedimentation of the macromolecular protein material contained in Keilin & Hartree's heart muscle oxidase preparation (see also p. 5). The

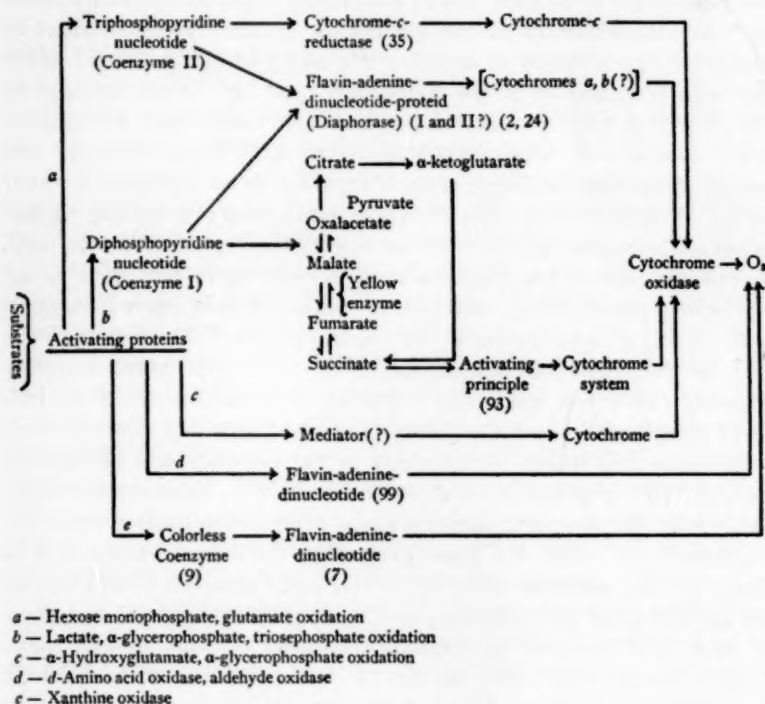


FIG. 2.—Respiratory mediators

mediator is a protein-like substance with a molecular weight of the order of 140,000; it is not identical with Straub's heart flavoprotein. It is probable, although not yet proven, that this mediator is inserted between the succinic dehydrogenase and the cytochrome system. It may be identical with the factor the removal of which in the experi-

ments by Stotz & Hastings (97) and in those by Hopkins *et al.* (39) led to partial or complete inactivation of their heart muscle oxidase preparations towards succinate.

The theory of Szent-Györgyi and his collaborators (103) on the catalytic function of the C_4 dicarboxylic acids and of their activating enzymes in the cellular respiration of pigeon breast muscle and of a number of other tissues may be considered well established from an experimental point of view. It is not clear, as yet, whether these compounds perform their task as respiratory mediators in conjunction with or independently of the diaphorase system. The extension of Szent-Györgyi's theory to include citric and α -ketoglutaric acid which has been proposed by Krebs & Johnson (see Fig. 2) continues to be the subject of a lively controversy. The observation (36) that minced heart muscle will form aerobically citric acid from pyruvate and malate (less from oxalacetic acid) is regarded as an argument in favor of the citric acid cycle. On the other hand evidence tending to disprove the operation of the cycle has recently been published. Breusch, in continuation of his previous work on the citric acid theory, has examined, quantitatively, the fate of oxalacetic acid upon incubation with freshly minced tissues of the cat and pigeon (21). He concludes that in the tissues examined the citric acid cycle plays no part. Thomas (109) has made the following observations which to him seem to militate against the theory of Krebs: (a) The conversion of oxalacetate into malate is the same in the presence and absence of oxygen; (b) in presence of arsenite the three C_4 dicarboxylic acids, viz., malic, fumaric, and succinic acids, are rapidly transformed into oxalacetic acid while the same process in the case of citric acid is slow; (c) the catalytic effect of the C_4 acids manifests itself within ten minutes after the beginning of the experiments whereas the effect of citric acid becomes appreciable only after a delay of one hour. Finally, Stadie *et al.* (88), on the basis of careful studies, conclude that citric acid behaves like a metabolite rather than a catalyst in muscle metabolism.

In conclusion, an interesting observation by Krebs & Cohen (54) may be mentioned because it suggests, in the opinion of these workers, that the system of glutamic acid \rightleftharpoons α -iminoglutaric acid might be a potential hydrogen carrier. They find that a mixture of α -ketoglutaric acid and ammonium salts, when incubated anaerobically with pigeon breast or heart muscle, will form glutamic acid, succinic acid, and carbon dioxide.

CATALASE

Purification and constitution.—Sumner and his co-workers have continued the study of catalase after examination in the analytical ultracentrifuge (102) of the crystalline preparation from beef liver showed it to be a homogeneous substance, very slightly contaminated with impurity. The total iron was found to be between 0.09 and 0.10 per cent (101). Contrary to the finding of Agner (4) in the case of chemically purified catalase preparations, the copper content of crystalline beef liver catalase was found to be not more than 0.0015 per cent. The maximum activity of such preparations is now stated to be as high as Kat.f. 35,700. The study of the blue substance liberated from catalase by treatment with acid acetone or hydrochloric acid plus glacial acetic acid led these workers to the conclusion that it is not identical with biliverdin and not proven to be verdohemochromogen although it is somewhat similar to both substances. This blue-green compound was first observed in 1935 during the acid cleavage of chemically purified horse liver catalase preparations (91). At that time it was identified, with the aid of Dr. R. Lemberg, as biliverdin; no opinion was expressed as to the importance of this substance for the activity of the enzyme. Sumner & Dounce, on the other hand, after confirming this observation, were of the opinion that the blue substance, or rather its precursor, represents a "second side chain" of the enzyme molecule (101) in addition to protoferriheme (Zeile, Stern). The blue substance is stated to contain iron, the total iron in catalase being distributed in approximately equal parts between the heme and the blue substance (101). This would suggest that one catalase molecule contains two bile pigment-like groups and two heme residues. This aspect of the catalase problem has also been studied by Lemberg and his associates. Spectroscopic evidence was offered to show that the biliverdin liberated upon acid cleavage of enzyme preparations is derived from a bile pigment hematin-protein, similar to choleglobin or verdohemochromogen (60). More recently, a quantitative analysis of crystalline ox liver catalase preparations led to the conclusion that catalase contains one, rather than two, groups of bile pigment hematin (verdohematin) and three, rather than two, groups of protohematin per molecule (59). The authors support Sumner's belief that the bile pigment hematin represents an essential part of the enzyme rather than an impurity or a product derived from the enzyme by hydrogen peroxide in a side reaction.

Crystalline catalase preparations have now also been obtained from horse liver (28) by a modification of the method used in the case of beef liver by Sumner & Dounce. The examination of a once-recrystallized sample showed an activity, Kat.f., of 50,000 to 55,000, a hemin content of about 0.9 per cent which agrees with the figure given for ultracentrifugally purified horse liver catalase (96), and a practically negligible copper content. The total iron content of 0.2 per cent indicates a contamination of the crystals with a small amount of an iron-rich protein previously isolated from horse liver by Lauffer and by Stern & Wyckoff (96). This particular sample contained a very small amount of bile pigment iron complex.¹⁰ In many samples of crystalline horse liver catalase prepared since then the ratio of bile pigment iron to hematin has varied from the value found in crystalline beef liver catalase to almost zero. Activities varying from about Kat.f. 30,000 to 55,000 were found, the preparation with Kat.f. 30,000 showing a ratio of bile pigment iron to hematin iron similar to that found in beef liver catalase. The diffusion curve of this sample, according to measurements of V. L. Frampton, indicated monodispersity. The group at Cornell now concedes that the blue substance formed from catalase upon treatment with acetone-hydrochloric acid is biliverdin (dehydrobilirubin).

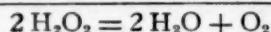
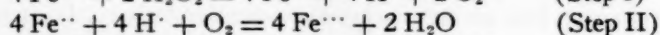
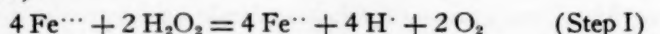
It is now evident that, at least in the case of crystalline horse liver catalase, no reproducible and constant ratio exists between bile pigment and hematin in different enzyme preparations. This throws considerable doubt on the earlier statements (see above) that the enzymatic activity is linked with a molecule containing heme and bile pigment groups in a fixed ratio. Instead it appears more probable to the writer that the enzymatic activity depends on the integrity of the heme structure and that the presence of bile pigment is the result of a partial breakdown¹¹ of active and native catalase along the lines

¹⁰ Personal communication from Dr. A. L. Dounce.

¹¹ The oxidative fission of the porphyrin ring system at one of the methine (—CH—) bridges between the pyrrol nuclei would be expected to yield a bile pigment iron protein which has the same protein moiety as catalase. This degradation of the hemin might conceivably take place in the liver *in vivo* which would explain why the same preparative procedure yields catalase preparations containing varying amounts of hematin and of bile pigment hematin. It is a moot question whether, in addition to molecules of the composition heme₄-protein and verdohematin₄-protein, there exist also molecular species of a mixed type such as heme₃-verdoheme₁-protein, etc., and whether such mixed or intermediate forms still possess some catalase activity.

indicated for the case of hemoglobin by Lemberg (58). Agner (5) has continued his efforts to purify catalase by electrophoresis. He claims to have obtained a preparation which proved to be homogeneous both in an electrical and in a gravitational field. The activity was Kat.f. 62,000 to 63,000. In contrast to his previous suggestion (4) that copper in the form of a conjugated protein might be an essential component of the active catalase system the author has now satisfied himself that the quantitative removal of the copper with dithiocarbamate does not seriously impair the activity of the enzyme. The further purification of chemically purified horse liver catalase by the application of Beams' air-driven ultracentrifuge and of Tiselius' electrophoresis separation cell has been pursued in the reviewer's laboratory along the lines indicated by the previous work with Wyckoff (96). By a combination of the two techniques catalase preparations have been obtained which are free from the iron-protein ferritin and which are homogeneous both upon electrophoretic and ultracentrifugal analysis (89). Even the most highly purified fractions contained bile pigment hematin. The identity of the bearer protein in the fully active catalase and the partly degraded chromoprotein would account for this and similar observations. The situation may be summed up by stating that catalase appears to be a protoferrheme protein and that attempts to correlate the enzymatic activity with other constituents, such as bile pigment-hematin or copper, have thus far failed. Ultimate proof for the constitution of catalase could best be furnished by its synthesis from pure protoferrheme and the native protein component. A prerequisite for this would be the preparation of the protein moiety in undenatured form. However, Agner's claim (3) to have accomplished a reversible dissociation of catalase by a procedure similar to that used by Theorell in the case of the yellow enzyme could not be confirmed in three different laboratories (Tauber, Sumner, Stern), and Agner has thus far failed to either substantiate or withdraw his claim.

Mechanism of catalase action.—The scheme proposed by Keilin & Hartree (45):



was essentially based on the finding of these workers that the decomposition of hydrogen peroxide does not proceed in the absence of

molecular oxygen. According to this scheme the ferri form of the enzyme is reduced by the substrate, hydrogen peroxide, and the ferrous form is then reoxidized by molecular oxygen. These conclusions as well as the experimental evidence on which they are founded have been contested during the past year. The observation (40) that luminous bacteria and preparations obtained from them will decompose hydrogen peroxide even at oxygen tensions too low to maintain the luminescence of these organisms (less than 10^{-5} atm. O_2) and the conclusion that oxygen is not required for catalase action have been criticized by Keilin & Hartree (49) on the ground that these observations were of purely qualitative character and that the experimental conditions (such as pH and salt concentration) were not conducive to complete inhibition of the enzyme. Weiss & Weil-Malherbe (116) state that they are unable to confirm the inhibition of catalase by removal of oxygen with the aid of the manometric technique employed by Keilin & Hartree. Furthermore they raise theoretical objections against the reaction scheme shown above.

In reply Keilin & Hartree (50) attribute the failure of Weiss & Weil-Malherbe to confirm their results to a defect in the arrangement of the experiments and to the use of impure enzyme preparations. The spectroscopic evidence cited in favor of the reaction scheme given above is considered unequivocal, and the fact that the results of Keilin & Hartree do not agree with certain theoretical considerations of Weiss & Weil-Malherbe is taken to suggest that these considerations require some revision. It is claimed that catalase, like the respiratory ferment and probably polyphenol oxidase, reduces oxygen directly to water upon reoxidation of the ferro form instead of forming hydrogen peroxide.

FREE ORGANIC RADICALS AS INTERMEDIATE STEPS IN OXIDATION

Almost a decade has passed since the problem of monovalent oxidation and reduction in biological processes arose in the wake of two different lines of investigation. In 1931, Friedheim and Michaelis as well as Elema encountered independently an intermediate of the nature of a free radical during the potentiometric study of pyocyanine and, in the same year, Haber & Willstätter published their notable paper on the rôle of monovalent-radical chain reactions in biological oxidation reactions. The recent conference on "Free organic radicals as intermediate steps in oxidation," held under the auspices of the

New York Academy of Sciences, provides a timely occasion to appraise the present status of the subject. In the present discussion it is proposed to draw not only upon the material presented at that conference by Michaelis (72), Wheland (118), Müller (75), and Schubert (85) but also on other data pertinent to the subject. For documentation and individual references the reader is referred to the reviews of Michaelis (70) and Barron (14) as well as to a recently published monograph on biological oxidation (81).

Any attempt to merge the theories of Warburg and of Wieland into a unified theory of biological oxidation had to take into account that the catalytic action of Warburg's and Keilin's iron-porphyrin systems is due to a cyclic change between the ferri and ferro form, i.e., to the loss and uptake of single electrons, whereas the reactions catalyzed by dehydrogenases were invariably formulated by Wieland, Thunberg and others as processes involving the simultaneous transfer of two hydrogen atoms or electrons. If both formulations were correct and if the iron catalysts were to join hands with the dehydrogenase systems somewhere in the chain of cellular oxidations, then such an interaction would postulate a trimolecular reaction since two ferri atoms would be required for the simultaneous acceptance of the two electrons from a dehydrogenase-substrate complex. This improbability may be overcome by two different assumptions: (a) There is interposed as a coupling link between the monovalent iron systems and the bivalent dehydrogenase systems a substance which is capable of exchanging one or two electrons at a time, or (b) the dehydrogenations previously formulated as bivalent processes are in reality composed of two monovalent steps. Thus, Haber & Willstätter formulated them as chain reactions involving the formation of intermediate monovalent radicals. A more recent development has led to the consideration of several coenzymes as substances capable of intermediate radical formation (see below). Inorganic model reactions, such as the catalysis of the $Ce^{++++} - Tl^+$ interaction by manganese which can lose and accept one or two electrons, have prompted Shaffer (cf. 86) to formulate his "valence harmony" hypothesis which encompasses, as a special case, the possibility mentioned under (a). Under these circumstances the experimental demonstration of the ability of naturally occurring substances to form radicals of an intermediate state of oxidation-reduction was bound to arouse keen interest and much speculation.

Several experimental techniques have been employed in the quest

for such intermediates, with varying measures of success. The most successful and most frequently used technique in this field has been and still is the potentiometric method which served in the pioneering work of Michaelis and Elema. Up to 1931, the theory of oxidation-reduction potentials as developed mainly by Clark and Michaelis covered only those cases where the titration curves correspond to the exchange of one or of two electrons simultaneously during the oxidation or reduction process. In fact, up to that time there were no experimental indications that any other cases existed. The very first titrations performed on pyocyanine in moderately acid solutions did not only reveal the formation of an intermediate form by the appearance of a color different from that of the fully oxidized and reduced forms but they also yielded titration curves with an "atypical" slope which could not be described by a one- or two-electron exchange; in still more acid solutions they showed a "break" near the midpoint. Michaelis and Elema were quick in grasping the significance of these observations and in filling the obvious gap in the theory of oxidation-reduction potentials. Today it is possible to deduce certain fundamental properties of such intermediate forms and to estimate their concentration from the slope of the titration curves with the aid of the so-called index potentials even in the absence of drastic color changes or when the break in the titration curve cannot be realized owing to the instability of the system at extreme pH values. The initial reluctance on the part of many chemists to accept the conclusions of Michaelis and of Elema as to the radical nature of the intermediate forms observed by them in the study of reversible organic dyestuffs was largely due to the fact that their potentiometric experiments were performed in aqueous solutions and that all other free radicals known to that time were either only stable in organic solvents, as for instance the radicals of the triphenylmethane type studied by Gomberg and Schlenk, or they were very short-lived under any conditions. This attitude was ultimately overcome by the unequivocal proof of the radical nature of these intermediates by the establishment of their paramagnetic nature, in other words by the demonstration that they have an odd number of electrons in their outermost (valency-determining) electron shell. The applicability of the magnetic method, however, is considerably more limited than that of the potentiometric method; it is restricted to those few instances where the intermediate can be isolated in solid form and free from the other components of the system (Kuhn, Katz) or where the dyestuff is sufficiently soluble to allow

for the detection of the paramagnetic nature of the intermediate by a significant decrease in the diamagnetism of the entire system in the course of the oxidation or reduction process (Michaelis *et al.*). Additional technical difficulties are presented by the necessity to employ very sensitive magnetic balances and by the limited choice of reductants which will cause a sufficiently slow reaction under all environmental conditions as to permit the performance of "magnetic titrations." The latest addition to the techniques available for the study of such intermediates is the polarographic method as first applied to the problem by Baumberger & Müller (cf. 75). The important question of the dimerization of such intermediates under certain conditions may be accurately studied by potentiometric means; valuable evidence of a qualitative nature may, however, frequently be gained by simple dilution tests. The stability of such radicals may be ascertained by studying spontaneous potential drifts or by simple spectroscopic and visual color observations (cf. 73). The flow method of potential measurements which was developed by Clark and Ball for the study of unstable systems of the epinephrine type should find a fruitful field of application here.

The formulation of these intermediates depends somewhat on the type of mechanism assumed in the reduction-oxidation process. If we consider the fully reduced or leuco form of a given system as the dihydro form then the intermediate is to be designated as the monohydro radical. If, on the other hand, the entire cycle is portrayed as the loss and gain of electrons, then the intermediate possesses one electron less than the fully reduced form and one electron more than the fully oxidized form. The exact characterization is determined by the chemical structure of the system and by the prevailing hydrogen ion concentration. Michaelis has proposed the term "semiquinone" for such intermediates. In the case of cationic dyes the semiquinoid intermediate is most stable in very acid solutions where it exists as a cationic radical; with anionic dyestuffs the opposite is true. The semiquinoid radical is at all times in equilibrium with the fully reduced (benzenoid) and the fully oxidized (quinoid) form. Establishment of the equilibrium is reached instantaneously; its position depends on the formation constant and dismutation constant of the intermediate. A radical may disappear reversibly not only by dismutation but also by di- or polymerization. It may, of course, also decay irreversibly by interaction with the solvent. There are instances, e.g., in the class of dipyrindylum complexes or in that of aromatic *p*-diamines (Wur-

ster's dyes), where the intermediate exhibits a higher degree of stability than the corresponding fully reduced, or, conversely, than the fully oxidized form. The generally remarkable stability of semiquinoid radicals is explained by Michaelis on the basis of quantum mechanics as due to a state of "resonance" between two possible, tautomeric configurations. The resonance is portrayed as the oscillation of the odd electron between two symmetrical points of the molecule, e.g., two nitrogen atoms in para position in a heterocyclic ring system. This basic requisite limits the possibilities of stable radical formation.

A survey (cf. 72) shows that semiquinoid radicals have been observed during the oxidation of aromatic diamines and the reduction of suitable paraquinones (e.g., duroquinone), orthoquinones (e.g., phenanthrenequinone sulfonate), phenazine and many of its derivatives (e.g., pyocyanine, safranine and rosindulin), isoalloxazines (e.g., riboflavin), indamines and indophenols, thiazines (e.g., methylene blue), and benzoine. The experimental conditions under which the semiquinone formation constant is sufficiently large for detection of the radical vary from case to case. It may be added that semiquinone formation is indicated from potentiometric titrations, although not proven, in the biologically occurring pigments hallachrome and toxoflavin, the chemical constitution of which is not yet fully established. A tabulation of the data available about semiquinone formation will be found in the recent article by Roman (84).¹² It will be seen that such radicals have been demonstrated in all of the more familiar classes of reversible bivalent oxidation-reduction systems. The inability to detect semiquinone formation in other systems, such as alcohol-acetaldehyde for example, explains, according to Michaelis, why such systems behave essentially as irreversible ones. The significance of these findings has been succinctly described by Michaelis (71).

The general acceptance of this theory for biological oxidation processes hinges obviously on the question as to what extent the existence, under physiological conditions, of semiquinoid intermediates in biological systems may be considered proven. It so happens that the color and concentration of all semiquinones thus far found become sufficiently pronounced for unequivocal measurements in the strongly acid or strongly alkaline regions. The existence of these

¹² Potential values and other data concerning biological and synthetic oxidation systems will be found in an earlier table (90).

radicals around pH 7 has been inferred either from mixed colors which are difficult to interpret in an exact manner or from the value of the so-called index potential, obtained from titration curves, and from the extent of its deviation from the theoretical index potential for a one-step bivalent system. This theoretical potential is 14.3 mv. at 30° and the deviations experimentally observed are often as small as 0.1 mv. which comes uncomfortably close to the error of the potentiometric experiments and of their graphical plot. In other cases, the difference is well outside the limit of error. Thus, with riboflavin, the index potential at pH 7.10 is 16.0 mv. from which value Michaelis *et al.* have calculated that at 50 per cent reduction about 10 per cent of the total dye can exist as the semiquinone. It should be pointed out that, as long as we can be certain of the existence of a semiquinoid form under a given set of conditions and in a given system, the absolute amount formed is of little importance provided that the formation constant of the semiquinone is not too small. Only in the latter instance will the concentration of the radical become so small as to constitute the limiting factor of the reaction rate (72). Unfortunately, however, a number of the most important components of biological oxidation systems, viz., the coenzymes, do not seem to be amenable to an exact potentiometric analysis. The normal potential of cozymase, for instance, appears to be so negative as to lie almost in the region of the hydrogen electrode.¹⁸ The potentials drift during the titration, and the suggestion that pyridine coenzymes are capable of semiquinone formation rests thus far only on the appearance of a yellow intermediate upon reduction and some non-conclusive spectrophotometric evidence. The same is true for thiamin (94). Perhaps the application of the magnetometric or of Roughton & Hartridge's flow method will ultimately yield definite information in these important cases.

Much speculation has been aroused by the interesting observation of Haas (33) that upon reducing the "old" yellow enzyme (riboflavin phosphoric acid protein) by hyposulfite in the presence of coenzyme II (triphosphopyridine nucleotide) at pH 7 a red intermediate is formed with the absorption spectrum of the cationic radical of the flavin instead of that of the green semiquinone ordinarily formed around pH 7. It has been suggested that this red intermediate

¹⁸ A recent determination with indicator dyes and milk flavoprotein as the mediator, however, indicates an E° value of -0.26 v. at pH 7.2 (10).

is a combination of the protein of the yellow enzyme with two prosthetic groups, i.e., the flavin in the semiquinoid state and the pyridine coenzyme. Such a fact, if well established, would obviously be of signal importance for the theory of biological oxidation. However, it must be stated that Haas has elaborated no further on his preliminary note and attempts in other laboratories to reproduce the phenomenon with the aid of coenzyme I (diphosphopyridine nucleotide) have failed.

FLAVOPROTEINS

The recognition of the flavoprotein nature of the widely distributed enzyme (diaphorase, coenzyme factor) which catalyzes the oxidation of dihydropyridine coenzymes by suitable acceptors such as methylene blue, but not molecular oxygen (24) has been confirmed by Euler and his associates (2). This clarifies the situation considerably and permits us to draw the conclusion that the physiological reoxidation of reduced coenzymes is in many instances brought about by alloxazine proteids (see also pp. 15-18). The action of these flavoproteins appears to be highly specific not only with regard to the hydrogen acceptor but also to the donator. Adler *et al.* (2) report that two different diaphorases occur in animal tissues, diaphorase I which reacts specifically with coenzyme I (diphosphopyridine nucleotide) and diaphorase II which dehydrogenates coenzyme II (triphosphopyridine nucleotide). The latter has not yet been shown to be inactive towards coenzyme I. Both groups of workers agree also that the colored group in diaphorase (I and II) is a flavin-adenine-dinucleotide. The latter is either identical with or closely related to the prosthetic group of Warburg & Christian's *d*-amino acid oxidase preparation as shown by an exchange of bearer proteins (98). The chemical constitution of this important coenzyme has been studied by Abraham (1). Upon acid hydrolysis the liberation of adenosine-5-monophosphoric acid and of riboflavin-5-phosphoric acid was demonstrated. This agrees with the view of Warburg & Christian that the coenzyme is a dinucleotide formed from adenylic acid and flavinphosphate and that the molecule is therefore built in a manner analogous to that of cozymase.

A simplified procedure for the isolation of the "old" yellow enzyme (flavinphosphate-proteid) from yeast has been reported from Kuhn's laboratory (117).

Xanthine oxidase, according to Ball (7), is a flavoprotein. The

prosthetic group, although being able to replace the coenzyme of amino acid oxidase and therefore representing undoubtedly a flavin-adenine-dinucleotide, does not seem to be identical in every respect with the active group of the amino acid oxidase. There are indications that the enzyme preparations contain, in addition to the flavin group, a colorless coenzyme which may be separated from the protein by dialysis (9). The experiments by Dixon and his collaborators (26) had led them to the conclusion that the enzymes in liver and milk which oxidize xanthine and aldehydes are identical. Ball found his purified preparation to attack both types of substrates, although it was irreversibly inactivated while oxidizing aldehydes.¹⁴ It is, therefore, of considerable interest that another flavoprotein has now been isolated from pig liver (99) which is stated not to be identical with xanthine oxidase but which will specifically oxidize aldehydes to the corresponding acids; molecular oxygen serves as the acceptor. The turnover number is 555, i.e., one enzyme molecule will oxidize 555 substrate molecules per minute at 38°. The flavin is present in the form of alloxazine-adenine-dinucleotide. However, there appears to be some colored grouping other than flavin associated with the enzyme molecule.

Some time ago F. G. Fischer (29) demonstrated the presence of an enzyme in yeast which catalyzes the hydrogenation of fumaric acid to succinic acid and which was believed to be different from succinic dehydrogenase which can act in the same manner under certain conditions. Further studies on this enzyme have now led to its recognition as an alloxazine proteid (30). It is possible to separate the prosthetic group from the native bearer protein by Warburg's method of acid dissociation in ammonium sulfate solution. Upon adding pure alloxazine-adenine-dinucleotide to the protein the full enzymatic activity is restored within a few hours.

BREAKDOWN OF PYRUVIC ACID

Pyruvic acid occupies a unique position among the intermediates in cellular metabolism. It plays an important role in most forms of carbohydrate breakdown, in transamination, in the formation of the so-called ketone bodies, and in many other processes which occur in

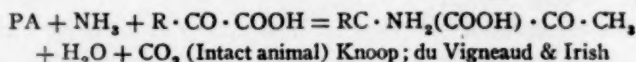
¹⁴ The same preparation will also catalyze slowly the reoxidation of reduced diphosphopyridine nucleotide by molecular oxygen or suitable reversible dyes (10).

plant and animal cells, as well as in microorganisms, under a variety of environmental conditions. This simple keto acid is preeminently fitted for this central role by virtue of its extraordinary reactivity, particularly if it is "activated" by suitable enzyme systems. A number of reactions of biological interest involving pyruvic acid is indicated in Table I (see also scheme on p. 34).

TABLE I

BIOLOGICAL REACTIONS INVOLVING PYRUVIC ACID*

Reaction	Material	Conditions	Authority
PA \rightarrow Acetaldehyde + CO ₂	Yeast	Aerobic, Anaerobic	Neuberg
PA + 5/2 O ₂ \rightarrow 3 CO ₂ + 2 H ₂ O	Brain	Aerobic	Peters, Long
PA + 1/2 O ₂ \rightarrow Acetic acid + CO ₂	{ Brain Gonococcus	Aerobic Aerobic	Peters, Barron
2 PA + H ₂ O \rightarrow Lactic acid + Acetic acid + CO ₂	{ Gonococcus Brain	Anaerobic Aerobic	Krebs Long
PA + 2 H \rightleftharpoons Lactic acid	{ Muscle Tumor	Anaerobic, Aerobic Anaerobic, Aerobic	Meyerhof Warburg
PA + Oxalacetic acid \rightarrow Citric acid	Muscle	Anaerobic	Krebs
2 PA \rightarrow Acetic acid + Formic acid	Streptococcus	Anaerobic	Barron
PA + Glutamic acid \rightarrow Alanine + α -Ketoglutaric acid	Muscle	Anaerobic	Braunstein
2 PA \rightarrow Succinic acid \rightarrow Fumaric acid \rightarrow Malic acid \rightarrow Oxalacetic acid \rightarrow PA + CO ₂	Kidney cortex	Aerobic	Elliott
PA + H ₂ O ₂ \rightarrow Acetic acid + CO ₂ + H ₂ O	Pneumococcus	Aerobic	Sevag
2 PA \rightarrow Acetoacetic acid	Liver	Aerobic	Embden
2 PA \rightarrow β -Hydroxybutyric acid	Muscle	Anaerobic	Krebs
2 PA \rightarrow Succinic acid	Testis	Anaerobic	Krebs



* For brevity in the table pyruvic acid is designated as PA.

A discussion of the role of pyruvic acid in biological oxidation will be found elsewhere (14, 81). Here only those reactions will be men-

tioned, the study of which has materially progressed during the past year.

The outstanding advance consisted in the reduction to a common denominator of such seemingly utterly different processes as anaerobic decarboxylation to form acetaldehyde, oxidative decarboxylation to acetic acid, and anaerobic dismutation to lactic acid, acetic acid and carbon dioxide. It could be shown conclusively that the same substance, viz., diphosphothiamin, functions as a coenzyme in all these instances and it was rendered very probable that in all these reactions this coenzyme undergoes a reversible oxido-reductive cycle.

An important study on the metabolism of pyruvic acid by animal tissues and bacteria has been published by Barron & Lyman (15). This paper represents an extension of the earlier experiments by Barron & Miller on the enzymes in gonococci that attack lactic acid (α -hydroxyoxidase) and pyruvic acid (α -keto-oxidase). It is shown that the orientation of pyruvic acid metabolism is, among other things, a function of the oxygen tension of the environment. In the enzymatically simplest systems devoid of α -hydroxyoxidase (lactic dehydrogenase), e.g., hemolytic streptococci, pyruvic acid is oxidized to acetic acid and carbon dioxide in oxygen and dismuted to acetic acid and formic acid in nitrogen. In more complicated systems, e.g., gonococci, which possess also α -hydroxyoxidase, the aerobic process again yields acetic acid and carbon dioxide while the anaerobic dismutation leads to acetic acid, lactic acid and carbon dioxide (Krebs). Barron was able to disprove the view of Krebs that the aerobic oxidation of pyruvic acid is connected with the anaerobic dismutation in such a manner that the lactic acid formed in the dismutation process is further oxidized by molecular oxygen. Instead, the aerobic process must involve a direct oxidative decarboxylation of pyruvic acid. The relationship between oxidation and dismutation of pyruvic acid, called by Barron & Lyman the "oxydismutation coefficient," in each cell is a function of the oxygen tension. Both reactions require diphosphothiamin as a necessary factor.

Lipmann has continued his work on pyruvic acid oxidation in lactic acid bacteria (*B. delbrückii*) with interesting results. Thus it was found that the dehydrogenation of pyruvic acid is coupled with a phosphorylation of adenylic acid (63). The process affords another source of phosphorylation energy for muscle tissue and appears to be analogous to the reactions of pyridine nucleotides in fermentation which have been elucidated by Meyerhof, D. M. Needham, Parnas,

Euler, and others. The donator of the phosphoric acid is probably the coenzyme of pyruvic dehydrogenase, viz., diphosphothiamin, which in turn is regenerated by an enzymatic synthesis from free thiamin and inorganic phosphate. The occurrence of such a synthesis has now been demonstrated in a variety of biological materials. It has recently been confirmed for liver and, to a lesser extent, for brain and muscle by Ochoa (78). In liver the synthesis depends on an active cell respiration.

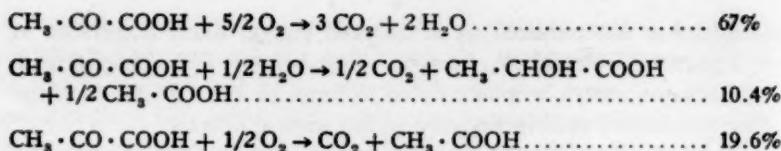
The anaerobic dismutation of pyruvic acid in *B. delbrückii* involves the participation of an alloxazine system as the coupling link (62). According to Lipmann (62, 63) the dismutation is a "fermentation-like" reaction with pyruvic acid-pyruvic dehydrogenase as the reductant, pyruvic acid-lactic dehydrogenase as the oxidant and flavin acting as the hydrogen carrier between the two enzyme systems. The reductant is the same both in aerobic dehydrogenation and anaerobic dismutation of pyruvic acid. The oxidants differ, depending on the experimental conditions: Oxygen acts as the oxidant in the aerobic process while pyruvic acid-lactic dehydrogenase takes its place in anaerobiosis. The alloxazine compound required for the aerobic oxidation of pyruvic acid has now been identified with the flavin-adenine-dinucleotide derived from Warburg & Christian's amino acid oxidase preparation (63a). The proteins involved in the two reactions are, of course, different.

Lipmann (64) advances the interesting hypothesis that the dehydrogenation of pyruvic acid may involve the intermediate formation of acetylphosphate. Such a hypothesis would provide an alternative explanation of the need for inorganic phosphate in the reaction; it would also account for the inertness of free acetic acid when it is added to systems capable of complete oxidation of pyruvic acid to carbon dioxide and water. The hypothesis is supported by the demonstration that upon incubating crude synthetic acetylphosphate with dry preparations of *B. delbrückii* and adenylic acid, roughly one-quarter of the latter is phosphorylated under the experimental conditions employed. Peters and his co-workers (79) incline to the view that not acetylphosphate but some other, as yet unknown, phosphorylated intermediate plays an important role in pyruvic acid metabolism in brain and bacteria. The existence of such an intermediate, these workers feel, is strongly suggested by the observation of Banga *et al.* (12) that adenylic acid is involved in a stage of pyruvate oxidation by brain tissue beyond that of oxidative decarboxylation. They find that acetyl-

phosphate is not oxidized by a dialyzed pigeon brain dispersion in the presence of phosphate, fumarate (see below), and adenylic acid. Furthermore, acetylphosphate failed to function as a donor of phosphate to adenylic acid in dialyzed rabbit muscle extract.

The group in Oxford has continued the active investigation of the mechanism of pyruvate oxidation in brain with particular reference to the role played by thiamin and diphosphothiamin. In some earlier work Peters had failed to substantiate the claim of Lohmann & Schuster that cocarboxylase (diphosphothiamin) exhibits an activity, of the same order as that of free vitamin B₁ (thiamin), in Peters' "catorulin" test system, containing slices of avitaminotic pigeon's brain. A careful reinvestigation (11) has shown that the apparently low activity of diphosphothiamin was due to the inability of the phosphorylated vitamin to reach the active centers concerned with pyruvate oxidation in brain. When fine brain dispersions are used in place of slices or "brei" the diphosphothiamin shows a very high catalytic activity. A maximum response is obtained with a concentration of $1.5 \times 10^{-7} M$; each molecule of the coenzyme catalyzes the uptake of 1500 molecules of oxygen per minute under optimum conditions. With fine brain suspensions, diphosphothiamin, but not monophosphothiamin, is much more active than free thiamin. The latter can exert its effect only after enzymatic phosphorylation in the tissue. At the same time it is shown that the C₄ dicarboxylic acid system of Szent-Györgyi is catalytically active in the oxidation of pyruvate by brain: small amounts of fumarate increase the oxygen uptake with pyruvic acid as the substrate, and malonate inhibits the catalysis. The fumarate may be replaced by malate or oxalacetate but not by citrate. This finding makes the participation of Krebs & Johnson's citric acid cycle in pyruvate oxidation in brain unlikely (12). Further analysis shows that the following components are necessary for the catalysis in brain: diphosphothiamine, fumarate, inorganic phosphate, adenylic acid, cozymase (12) and Mg⁺⁺ or Mn⁺⁺ (77). By analogy with Lipmann's experiments with bacteria, flavin-adenine-dinucleotide may have to be added to this list. The metal ions are probably concerned with the phosphate transfer to adenylic acid. The phase in which cozymase is involved remains to be elucidated.

The quantitative aspects of the pyruvate oxidation in brain have been examined by C. Long in Peters' laboratory (66). It was possible to account for 97 per cent of the pyruvic acid which disappears during the process by the following reactions:



Furthermore, the respiratory quotient of 1.28, as calculated from these data, agrees very well with the experimental value of 1.3 found by McGowan. On the other hand, as Long points out, the fact that a balance sheet can be drawn up in this way does not necessarily mean that all three processes occur independently of each other, or even that, as such, they take place at all. It is very unlikely that the first reaction actually proceeds in this "explosive" fashion, i.e., that it yields carbon dioxide and water without the formation of any intermediate. On the contrary, work from the same laboratory suggests an even more complex mechanism of pyruvic acid oxidation in brain than has hitherto been suspected.

The substitution of other keto acids for pyruvic acid in the brain system (67) had the result that pyruvic and α -ketobutyric acids were about equally rapidly attacked under aerobic and anaerobic conditions; α -ketovaleric acid was found much less reactive. Whereas the larger part of the pyruvic acid is completely burned to carbon dioxide and water (see below), the decomposition of α -ketobutyric acid was arrested after the oxidative decarboxylation, i.e., presumably at the propionic acid stage. This finding points to the existence of an initial common path for oxidative decarboxylation of the two keto acids and to the presence of a further enzyme system which can deal only with the intermediate arising in the pyruvic dehydrogenation.

Finely ground pigeon brain preparations of the type where diphosphothiamin exhibits a strong catatorulin effect may be separated into two fractions by centrifugation (13). The sedimented solid contains an active pyruvic dehydrogenase as shown by Thunberg experiments with methylene blue as acceptor. The supernatant solution contains, besides an active triosephosphate dehydrogenase, a system which upon addition to the sediment will induce rapid oxygen uptake with pyruvate as the substrate. The oxidation of pyruvate and the degree of its completeness depend on the presence of adenylic acid. The activating effect of fumarate has already been mentioned.

An investigation into the mechanism of the action of diphosphothiamin as cocarboxylase in the usual yeast test system has yielded

the following results (94). It is improbable that the decarboxylation of pyruvic acid by yeast carboxylase involves a "Langenbeck cycle," i.e., that the first step in the catalysis consists in the combination of diphosphothiamin with pyruvic acid to form a catalytically active substituted imino acid. On the other hand, the high biological activity of dihydrococarboxylase in the yeast test system (and also in avitaminotic pigeons) suggests that the coenzyme goes through an oxido-reductive cycle in the course of its action. Since the reaction proceeds at about the same speed under aerobic and anaerobic conditions, oxygen, as the acceptor of the coenzyme hydrogen, is to be ruled out. Inasmuch as the straight decarboxylation of pyruvic acid to form acetaldehyde does not constitute a change in the state of oxido-reduction of the substrate, it is difficult to visualize pyruvic acid as the reductant of the coenzyme, unless acetaldehyde is not the primary product of this interaction. The detailed formulation of the catalysis must wait until further evidence on the reaction mechanism becomes available. When free thiamin or diphosphothiamin is reduced at neutral reaction, the ensuing change in the ultraviolet absorption spectrum, while definite, is not as great as in the case of the pyridine coenzymes (69).

Recent experiments by Elliott and his colleagues (32) on the metabolism of lactic and pyruvic acids in ox retina and chick embryo indicate that the cycle of reactions previously postulated for kidney cortex, involving the successive formation of succinate, fumarate, malate, oxalacetate, and finally half the original amount of pyruvate, does not occur in the tissues studied. The same conclusion had been reached previously for tumor, brain, testis, and liver tissue. There is an indication that in retina the breakdown of lactic and of pyruvic acids proceeds along different lines.

NOMENCLATURE

The unsatisfactory state of affairs with regard to nomenclature in the field of biological oxidation and enzymes in general has already been mentioned in last year's review (26). The situation has become so acute that it is planned to devote a special conference to questions of terminology at the meeting of the American Societies for Experimental Biology at New Orleans (March, 1940).

To illustrate the difficulties that exist mention might be made of the confusion created by calling "carriers" the protein components

of enzymes as well as hydrogen-transporting systems (e.g., flavoproteins) and non-enzymatic intermediary catalysts (e.g., pyocyanine). Here the suggestion of Oppenheimer (80, see also Oppenheimer & Stern, 81) to designate the protein moiety of enzymes as the "protein bearer" and non-enzymatic intermediary catalysts such as thiols, ascorbic acid, and reversible dyestuff systems, as "mesocatalysts," may be found helpful.

The nomenclature in the field of intracellular hemochromogens is equally unsatisfactory. Thus the names cytochrome-*a*, -*a*₁, -*a*₂, and -*a*₃ suggest a chemical relationship which does not necessarily exist. The *a*₂ component, for instance, contains possibly a green hemin or a biliviolin as the prosthetic group while the others are probably pheohemin derivatives.

In general, the proposals made by Warburg (112) appear to fulfill the requirements of a rational terminology.

TECHNIQUE

Several new instruments have been developed which promise to yield valuable information when applied to problems relating to biological oxidations and reductions. A novel type of microrespirometer has been described (37) which permits the measurement of gas exchanges of the order of 1 c.mm. per hour with a systematic error not greater than that involved in the usual manometric methods. The biological material to be studied is enclosed in a glass chamber equipped with a mica diaphragm. The pressure changes taking place in the vessel are compensated by external pressure applied by a manometric device. The point of compensation is determined with the aid of a sensitive optical indicator system involving a pair of small mirrors attached to the mica diaphragm. The method has been applied to a study of the metabolism of small amounts of normal and malignant tissues (17), and to a comparison of the metabolism of normal skin epithelium of the rabbit with that of the Shope papilloma (18). The authors find closely agreeing values for both tissues and state that neither glycolysis nor a low respiratory quotient of glycolyzing tissue can be considered as typical of tumor metabolism.

The principle of the Cartesian diver which has recently been employed as an ultramicromanometer for the study of enzyme reactions by Linderstrøm-Lang and his co-workers has now been adapted to the examination of the metabolism of minute quantities of surviving

tissue (19). The technique has thus far been used in extended studies on the metabolism of the various regions of the amphibian gastrula (19, 20).

An ingenious arrangement which may be used either as a constant volume differential manometer or as two simple manometers has been described by Summerson (100).

The photoelectric reaction meter of G. A. Millikan, which embodies the flow tube principle of Roughton & Hartridge, has been modified in two ways. B. Chance,¹⁵ in collaboration with Roughton and Millikan, has replaced the conventional arrangement, where the light transmission of the fluid in the observation tube is measured by a photoelectric cell while the reaction mixture streams through it at a constant rate, by a device where the distance of the photoelectric cell from the point of mixing remains constant. Instead, the flow rate is accelerated in a defined manner which is equivalent to a shift in position of the photocell. The reaction curves are obtained by photographing the screen of a cathode ray oscillograph; they are then referred to a derivative time base. D. DuBois (unpublished), on the other hand, in collaboration with the present writer, has constructed an automatic micro-flow machine which shares some features with an apparatus developed previously by Thiel & Logemann. While the reaction mixture streams through the observation tube (made of polyacrylic resin) at a constant speed it is mechanically scanned by a photoelectric photometer employing monochromatic light. The changes in light transmission due to the reaction are recorded with the aid of an electrocardiograph galvanometer camera. A complete reaction curve may be obtained with as little as 1 cc. of fluid. Half-times of reactions down to 0.2 msec. may be determined. The apparatus is to be used in an extension of work on the formation of enzyme-substrate compounds and in an investigation of the rates of interaction of oxidation-reduction systems of biological interest.

SYMPOSIA AND REVIEWS

The centenary of the discovery of the cell by Schwann & Schleiden was commemorated by a symposium on cytology held under the auspices of Stanford University (to be published). One of the subjects discussed was that of cellular respiration systems. Papers were read by H. Theorell, A. v. Szent-Györgyi, and others. A conference on tissue respiration was held under the auspices of the

¹⁵ Personal communication.

British Association in Dundee. Papers presented at that occasion and other papers which were scheduled but not read owing to the imminence of the war have been reviewed by Peters (82). The field of biological oxidation in its various aspects was discussed by a group of over thirty workers at the Biological Laboratory in Cold Spring Harbor. The papers read there and the discussions following them will form the seventh volume of the series on Symposia of Quantitative Biology edited by the laboratory. The more special aspect of the significance of free radical formation during two-step oxidation processes was the topic of a conference arranged by the New York Academy of Sciences. The proceedings will be published in the Annals of the Academy.

Cellular oxidation systems have been discussed from a physicochemical point of view in a competent and lucid manner by Barron (14). Recent work on coenzymes was reviewed by Baumann & Stare (16). The properties and function of muscle hemoglobin have been discussed by Millikan (74) who has made notable contributions to the subject. Other useful review articles deal with respiration in the central nervous system (83), tissue respiration (68), and low-molecular natural oxidation-reduction systems (29). Oppenheimer has now completed the supplementary volume to his previously published reference work on enzymes (80). A monograph on biological oxidation has also been published (81).

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LABORATORY OF PHYSIOLOGICAL CHEMISTRY
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PROTEOLYTIC ENZYMES¹

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The enzyme chemist has always been interested in the proteins, because they were among his most important substrates. Now the protein chemist is also interested in enzymes because they are proteins. The proteinases thus invariably confront the investigator with at least two proteins at once, the enzyme and the substrate. And two proteins are even more difficult to consider than one. Lately, the development of knowledge regarding protein-splitting enzymes has had to take, in the main, two directions; the investigation of proteins undergoing enzymic hydrolysis and the investigation of the enzyme proteins that produce it. The first may properly belong to the protein chemist, the second is the more classical form of enzyme chemistry. But the line of demarcation is vague and rapidly getting vaguer.

A protein is now being regarded by many workers as a pattern rather than as a thing—a molecule of definite composition but of dubious size. For this, if for no other reason, analytical work on proteins is important. Methods for determining amino acids and for determining the extent of protein hydrolysis are therefore of more than passing interest to the enzyme chemist, although they have not yet reached anything like maximum development. Bergmann & Stein (1) have recently described the determination of glycine, alanine, leucine, and proline and the application of the methods to collagen and gelatin. It is obvious that such methods may be of great importance when applied to cases where proteolysis takes place. Submicro quantities of total nitrogen and of amino, amide, amine, and peptide nitrogen may be estimated by methods described by Borsook & Dubnoff (2) who used an electrometric titration to this end. It is interesting that peptide nitrogen is determined by enzymic hydrolysis with

¹ *Food Research Division Contribution No. 475.* The date to which the literature has been covered is December 1, 1939, but the difficulty in obtaining recent publications of foreign origin makes it probable that valuable contributions in this field have not received due recognition. The reviewer hopes that his apologies and regrets may be accepted in such cases.

an extract made from *Aspergillus wentii*. Tested against peptones this peptidase gave nearly the same results as those obtained by hydrolysis with strong acid. Application of the author's well-known micro methods for the estimation of enzymes in minute amounts of biological material, such as an eighth of a sea-urchin egg, have been described by Linderstrøm-Lang (3).

PARTICULAR CASES OF PROTEOLYSIS

The total number of peptide bonds in the molecule of crystalline lactoglobulin was found by Hotchkiss (4) to be very close to 341,² (assuming the accepted molecular weight of 39,000). On the other hand, Miller (5) has measured the peptide bonds split in crystalline lactoglobulin by crystalline trypsin and chymotrypsin. Trypsin was estimated to hydrolyze 33 to 35 linkages; chymotrypsin when used after trypsin extended hydrolysis to 47 to 50 linkages. Chymotrypsin alone split the protein as far as when it had been previously attacked by trypsin, that is to the extent of about 47 to 50 links. But trypsin following chymotrypsin extended this figure to 65 to 68 linkages. Some years ago Northrop & Kunitz (6) found with casein that although hydrolysis with chymotrypsin was more extensive than with trypsin, the sequence in which the enzymes were employed made very little difference to the total extent of hydrolysis. Evidently this is not the case with lactoglobulin. The two enzymes appear to attack, in the main, two different sets of bonds in casein. It may be, as the author points out, that there are an appreciable number of groups in lactoglobulin susceptible to both enzymes. This, however, hardly explains why the sequence of the enzymes affects the extent of total hydrolysis found after both enzymes have been used. It appears to the reviewer that chymotrypsin may prepare the lactoglobulin for tryptic digestion that otherwise could not occur. Such an effect might be due to a species of denaturation or it might be due to the greater digestibility by trypsin of the protein fragments formed by chymotrypsin.

The question of the possible denaturation of the protein during

² This does not quite fit with either Bergmann's or Wrinch's conception of the makeup of the protein molecule, but it is less favorable to the latter hypothesis.

digestion makes it difficult to be sure that results on native proteins are interpreted correctly. Native proteins are undoubtedly attacked very slowly, if at all, by crystalline enzymes under conditions where the protein is stable in the absence of the enzyme. Linderstrøm-Lang (7) has pointed out that while crystalline trypsin attacks globular proteins very slowly it also attacks synthetic peptides. The deduction that peptide bonds are present in globular proteins would lose its validity if the denaturation of the protein is a reversible reaction. It is evident that the denatured protein would be digested more rapidly than the native protein, and denaturation would continue in order to satisfy the equilibrium. But the experiment would represent the behavior of the denatured rather than of the native form. It is also pointed out that evidence for the reversible denaturation of proteins was presented by Anson & Mirsky some years ago.

This argument is supported by the experiments of Linderstrøm-Lang, Hotchkiss & Johansen (8) in which temperature coefficients for the digestion of lactoglobulin by (crude) trypsin were determined. By greatly increasing the quantity of enzyme, it was hoped that the rate of denaturation would prove to be the limiting factor in the rate of protein hydrolysis. The temperature coefficient for the hydrolysis would then approach that of the reversible denaturation, which is thought to be very high. A definite but small rise was found with a large quantity of trypsin. Brief heating also increased the rate at which the protein digested, but this rate receded again if the heated protein stood twenty hours before the enzyme was added, thus indicating a reversible denaturation. In the authors' opinion the experiments indicate that peptide bonds, like sulphydryl groups, may appear upon denaturation, but whether some are preformed in the protein molecule could not be decided.

Denaturation of the proteins in soybeans was found by Jones & Gersdorff (9) to take place simply on dry storage of the material, particularly in air. Increased digestibility was paralleled by the usual signs that the protein had become partly denatured.

Autolysis of proteins.—Practical application of enzyme reactions to the digestion of proteins is still rarer than one would expect, but two new uses of proteolytic enzymes seem of interest—the purification of diphtheria antitoxin by Sandor (10) and the preparation of arginine and ornithine by the pancreas enzymes as used by Hunter (11).

Experiments on autolysis, however, have interest not only as con-

tributing to knowledge of the nature of tissue proteins and the enzymes that hydrolyze them, but also because in the cold-storage of raw agricultural products, autolytic reactions are responsible for the major part of the breakdown that takes place. Lineweaver (12) has considered the energy of activation of enzyme reactions and their velocity below 0° , in comparison with other types of catalysis and with bacterial action. The speed of enzyme action is naturally decreased by lower temperatures, but not in the same degree as the speed of microbial action or of non-enzymic catalysis. In raw materials enzyme reactions are thus relatively important at low temperatures compared, for instance, to bacterial decomposition. Among the enzymes considered were the proteinases chymotrypsin and cathepsin. When the system froze, the activity of these enzymes dropped markedly with the change in state. It is not to be inferred, however, that all enzyme actions are equally affected by low temperatures or even by freezing. The soluble proteinases appear to be greatly affected, lipase far less.

The survival of cathepsin in autolyzing liver has been shown by Eder, Bradley & Belfer (13) to depend on the pH. Below pH 3 the enzyme was rapidly inactivated and the cessation of proteolysis below pH 3.5 is said to be determined more by failure of the enzyme than by lack of a substrate. Between pH 3 and 5 proteolysis was quite extensive and even at a more alkaline reaction the enzyme was inactivated slowly. The extent of autolytic changes in the liver proteins has been investigated by Luck, Eudin & Nimmo (14), with a technique that permitted autolysis to be followed beginning with perfectly fresh and blood-free tissue. Tyrosine liberation was found to proceed at the same rate as the splitting of peptide bonds. It is interesting that potassium iodate was found to inhibit, though it is difficult to say why it should unless one of the enzymes involved is inactivated by oxidation. It was formerly thought that the proteinase of liver, (cathepsin) was a sulphhydryl enzyme similar to papain and therefore inactivated by oxidation. The isolated enzyme, however, is now known not to behave in this way and shows no signs of inactivation by the reagents usually employed to identify the sulphhydryl enzymes. There is, however, reason to believe that the proteinase is accompanied by a peptidase which is activated by reducing agents and inactivated by oxidation. In this event the total proteolysis, which includes splitting of peptides, would be affected by the degree of oxidation. Maver (15) has made crude cathepsin from malignant and

from normal rat tissue by partial autolysis and precipitation with ammonium sulphate. Such precipitates contained both enzymes and substrates and underwent self-digestion. The addition of cyanide or cysteine accelerated both the autolysis and the digestion of added liver protein.

A new series of problems dealing in part with tissue enzymes has sprung from the work of Kögl & Erxleben (16, 17) on tumor and embryo tissues. By acid hydrolysis of tumor proteins, appreciable quantities of so-called "unnatural" amino acids,³ notably glutamic acid, have been obtained. Improved methods for isolating the racemic mixture of *dl*-glutamic acid have been worked out by Kögl, Erxleben & Akkerman (18).

Since the *d*-forms of amino acids appear to be built into tumor proteins,⁴ the possibility is evident that the molecule of protein may acquire an entirely different shape and also perhaps be no longer susceptible to attack by ordinary enzymes. Such an assumption helps to explain the growth of tumor tissue and the multiplication of virus proteins, but it does not help as yet to elucidate the genesis of the "unnatural" amino acids, nor whether special enzymes exist to decompose proteins containing them. This very new field has great potentialities. In any case, Kögl regards the degree of racemization as a criterion of the uniformity of tumor tissue and perhaps of its malignancy.

The non-hydrolytic breakdown of proteins.—Proteins may be broken down by heating to above 140° in non-aqueous solvents such as glycerine, β -naphthol, or acetic acid. A study of the products resulting from this type of decomposition has been made by Fodor and his collaborators (19). The protein fragments may be separated into

³ Throughout this section, the *l*-form of an amino acid refers to that optical variety occurring normally in nature, irrespective of the actual direction of its optical rotation. The so-called "unnatural" or *d*-form is now known to occur naturally in tumor tissue, and may also exist in normal tissues (besides embryo). In any case the distinction between "naturally occurring" and "unnatural" amino acids should now be made with this proviso in mind, as relative rather than absolute.

⁴ The importance that glutamic acid acquires in this picture of tumor tissue (in a sarcoma thirty-nine per cent of the glutamic acid found was of the *d*-variety) points again to its importance as a sort of key substance in amino-acid metabolism. It will be remembered that glutamic acid undergoes deamination in the presence of pyruvic acid, to form alanine—the transamination reaction of Braunstein & Kritzmann.

several fractions by precipitation with various concentrations of alcohol and in other ways. These fractions, which are called acro-peptides, were split by proteinases, but were not susceptible to digestion by peptidases. In the past, gelatin, casein, edestin, egg albumin, and the phospho-protein from yeast have been investigated in this way. Feigenbaum (20) has also investigated the breakdown products of fibrin in β -naphthol and has obtained fractions whose molecular weights seem to be quite low (700 to 900). Kuk (21) has studied the products obtained from casein, recovering about two-thirds of the original protein as material of low molecular weight. Lichtenstein (22) has reported that acro-peptides from casein, gelatin, and edestin were split by crude pancreas extracts and also by purified proteinase preparations therefrom, though by the latter to a lesser degree. These acro-peptides were also attacked by pepsin but not by yeast polypeptidase until after the action of a proteinase (pepsin, papain, or pancreas proteinase). The conclusions which in the main Fodor and his school draw from these experiments are that acro-peptides are closed chain complexes consisting of four or a multiple of four amino acids. Proteinases appear to open such closed polypeptide complexes and only thereafter are they susceptible to peptidase action. The closed peptide complex is regarded as the true basic unit of protein construction. The objection has naturally been made that such complexes are formed from the protein during the vigorous treatment that comprises the process of preparation. It is interesting, however, to note that open chain peptides subjected to the same treatment did not form closed chain complexes, and it must be admitted that the enzymic behavior of the acro-peptides is extremely curious and suggestive.

PARTICULAR PROTEOLYTIC ENZYMES

Pepsin.—More information concerning the active center in pepsin has been obtained. Felix & Mager (23) found that dialyzed pepsin inactivated by standing in dilute sulphuric acid or in a trypsin solution gave a precipitate with clupein methyl ester. This precipitate together with casein showed a slight digestion, whereas the clupein ester alone and the inactivated pepsin alone gave nothing. The experiment may indicate the transfer of some sort of active group from the original pepsin to the modified clupein.

It will be remembered that Herriott inactivated pepsin by iodine

and concluded that a substitution in the tyrosine of the enzyme protein was responsible for the loss of activity. Substitution undoubtedly occurs, for di-iodotyrosine was isolated from an experiment. A similar conclusion had also been reached by Herriott & Northrop studying the acetylation of pepsin. More recently Philpot & Small (24) found the partial inactivation of pepsin by nitrous acid to indicate that alteration of some of the tyrosine led to the loss of activity. Now, however, Philpot & Small (25) have measured the extent to which iodine was introduced into the tyrosine of pepsin and found (at pH 5.4) that some other group present in the protein reacted more rapidly with iodine than the tyrosine did. When this group has reacted the pepsin was inactive. The group is said not to be tryptophane, histidine, proline, or hydroxy-proline. Nor is it thought to be a sulphhydryl, amino, carboxyl, peptide, simple ketone, or simple phenol group. As the real nature of the group is so elusive, the authors think that it may be some unusual substance peculiar to pepsin. Details of the experiment have not yet become available, but in view of the earlier work it seems unlikely that nitrous acid acts on this iodine-absorbing group. It is plausible that a part of the tyrosine and the newly discussed group are both essential to the activity of pepsin.

Unfortunately for the interpretation of all such experiments, pepsin shares with papain the unenviable distinction that the ordinary crystals are definitely known to contain more than one substance. It has long been known that crystals of pepsin usually contain variable amounts of non-protein nitrogen, which may be a decomposition product of the enzyme itself. Furthermore, Tiselius, Henschen & Svensson (26) purified crystalline pepsin considerably by electrophoresis.

Steinhardt (27) has shown that the solubility of crystalline pepsin in a solution thereof increased with the amount already in solution. This again indicated that the material was not a single substance.⁸

Desreux & Herriott (28) by means of solubility diagrams have shown that pepsin when only partly purified contains at least three proteins, of which two possess proteolytic activity. The most soluble component was obtained pure and crystallized. The crystals were in-

⁸ The determination of solubility in the presence of increasing amounts of solid is one of the best methods now available to demonstrate the purity of an enzyme protein. Theoretically, the solubility of a pure substance is independent of the quantity of solid present. The behavior of quite a number of crystalline proteins, among them several enzymes, corresponds closely, though perhaps not quite exactly, to the theoretical requirement.

distinguishable from those previously described for pepsin. This material behaved as a homogeneous substance in the electrophoresis apparatus of Tiselius, and had a specific activity close to that previously obtained by the purification of pepsin in this apparatus (cf. 26). This form of pepsin is less stable than the less soluble variety. Since the proportions of the several components have been shown to vary from one pepsin preparation to another, differences in solubility, stability, and specific activity between pepsins are no longer surprising.

Solubility diagrams for pepsinogen were also made by the same authors (28) and also found to indicate the existence of several components. One pepsinogen present in small quantities yielded an unstable pepsin nearly twice as active as the pepsin previously obtained.

An interesting contribution to new crystalline enzymes is salmon pepsin, crystallized by Norris & Elam (29). The enzyme, prepared from the stomach mucosa of a Pacific Coast salmon was a protein of globulin type with an activity comparable to mammalian pepsin. The activity decreased normally with lowering of the temperature. Just as in the case of animal pepsin, the enzyme was found to exist originally as a zymogen convertible to the enzyme in acid solution. This is the first proteinase from a cold blooded animal to be purified by crystallization and its comparison with warm blooded pepsin cannot fail to be important.

Differences between the coagulating action of several milk-clotting ferments on Linderström-Lang's casein K₀ have been defined in one direction at least by the work of Lundsteen (30), who has shown that the enzymic change of casein to paracasein is optimal at pH 7 with chymotrypsin, at pH 5.35 with rennin, and at pH 5.25 with pepsin. After exposure of the casein to the enzyme at any desired pH, the system was brought to pH 4.86. If the enzyme action had been completed during incubation at the first pH, clotting took place within a few seconds after adjustment to 4.85—otherwise it required a much longer time.

Papain.—The nature of papain is a matter of interest because of the widespread occurrence of papain-like enzymes in the plant world. Similar enzymes have lately been obtained from lima beans (31), wheat flour (32), milkweed (33), and previously from pineapples and figs. They are also important because these enzymes constitute the only class of proteolytic ferments recognized by all hands to be reversibly oxidizable and reducible, and hydrolytically active (on all

substrates) only in the reduced state. The oxidation and reduction is thought by nearly all workers [contrary, cf. Okumura (34)] to represent the disappearance and reappearance of a sulphydryl group in the protein. This property is not only important in principle but has at times curious technical significance. Thus the aging of flour improves the quality of the bread made therefrom, because some of the proteinase has been oxidized during storage and cannot modify the flour proteins as readily as in new flour.

While the full action of papain is at the moment admitted everywhere to be done only by the reduced enzyme, Ganapathy & Sastri (35) offer data to show that oxidized papain still hydrolyzes egg albumin or gelatin but not certain peptones. By subsequent activation with reducing agents the specificity is extended to the peptones again. The authors conclude that the sulphydryl group is essential to peptone, but not to gelatin hydrolysis. The work was done with an alcohol precipitate of soluble protein from fresh papaya latex, not altogether comparable to crystalline papain, with which results of somewhat different character have been obtained.

Balls & Lineweaver (36) working with crystals of papain have found that the crystals may contain simultaneously three forms of the enzyme: an active form, an inactive form that may be activated by cysteine or cyanide, and an inactive form that cannot be activated thereby. The proportions of these three forms varied with the method of preparation. If the first crystallizations were carried out in the presence of cyanide or in the absence of oxygen, the proportion of active enzyme was greatly increased, and the proportion of irreversibly inactive enzyme very greatly diminished. Fresh latex, which is probably wholly active to start with, appears to contain a factor decidedly destructive to the activity of papain in the presence of air.

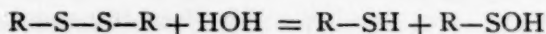
The ratios of protein-splitting to milk-clotting and to hippuryl-amide hydrolysis were however about the same in all the crystal preparations, irrespective of their intrinsic activity. This appears to indicate that the three activities undergo the same oxidation and reduction effects, and to about the same extent.

While the crystalline enzyme is definitely not the only milk-clotting catalyst in fresh latex, evidence has been found that a potential sulphydryl group is essential to the activity of crystalline papain (37). The oxidation of this group was however observed to present definite peculiarities. In the native protein it gave no satisfactory nitroprusside test, could not be titrated by porphyrindin and was rather resist-

ant to ferricyanide. When the protein was denatured the nitroprusside test became strongly positive. The group in the native protein reacted slowly to cystine and quantitatively and rapidly with iodoacetic acid. In the molecule of crystalline papain there appears to be only one potential sulphhydryl group responsible for the enzymic activity.

Crystalline papain, like a prolamine, is soluble in seventy per cent alcohol and without injury to the activity. It has a molecular weight of about thirty thousand.

Schöberl & Fischer (38) have found that cyanide, added to a partly purified preparation, produced greater activity than the original raw product could be made to show. They also found thioglycollic and thiohydracrylic acids to be most excellent activators after purification though not necessarily before. A very interesting observation by these authors is that zinc was very inhibitory to papain. Activation is produced not only by thioglycollic acid but in alkaline solutions (pH = 10.4) by dithiodiglycollic acid. In order to explain how an oxidized sulphur compound can activate the enzyme, the authors call attention to a hydrolytic reaction of dithiodiglycollic acid at alkaline pH:



Under alkaline conditions, a reducing substance titratable by iodine was actually formed during the enzyme activation.

Considerable interest has been evinced for a number of years in the reported ability of oxidized papain to synthesize protein-like substances from the digests produced from protein by reduced papain. Strain & Linderstrøm-Lang (39) were unable to find such an effect, but Maver & Voegtlin (40), repeating former work of theirs, claim that special conditions for observing the synthetic action must be rather rigidly adhered to. In a system composed of fibrin, papain, and glutathione these authors found that the hydrolysis of fibrin was reversible after oxygenation, but only when high concentrations of fibrin were used. Reversibility was determined by an increase in protein nitrogen.

The tryptic enzymes.—The hitherto disputed action of kinase in the activation of trypsinogen has now been cleared up by Kunitz. This followed an investigation of the activation of the pancreatic zymogens, trypsinogen and chymotrypsinogen, by means of trypsin itself (41). A new enzymic action of crystalline trypsin was thus

brought to light, namely the transformation of crystalline trypsinogen into an inert protein that cannot thereafter be changed into trypsin protein by any of the known activators. Thus during the activation of trypsinogen by trypsin two simultaneous processes occur. Some of the trypsinogen is changed into trypsin, the remainder into the inert protein. The transformation into inert protein is catalyzed by trypsin and the process follows the course of a monomolecular reaction, the velocity being proportional to the concentration of trypsin (the catalyst) and to the concentration of trypsinogen (the substrate). The proportion of trypsin and inert protein, the two end products formed from the trypsinogen, depends on the pH. Inert protein is formed faster in the alkaline range; in the acid range trypsin is more readily formed. The activation of trypsinogen by kinase apparently proceeds in a similar way. This was first investigated by a kinase prepared from a penicillium mold. The trypsin formed is identical with that obtained by the spontaneous autocatalytic activation of trypsin at pH 8, but the action of the mold preparation occurs at pH 3 to 4, where it follows the course of a monomolecular reaction (42). The kinase, in this instance, is a catalyst.

The activation of trypsinogen by hog kinase was then studied with a very highly purified preparation of enterokinase. This was prepared from the fluid contents of the duodenum by methods that included acidification to pH 4, whereby most of the undesired material was removed, then fractional precipitation with ammonium sulphate at pH 8. Subsequent salt fractionation of the latter precipitate resulted in an extremely powerful preparation of enterokinase (43). A method of estimating kinase by the use of crystalline trypsinogen was also worked out.

It must be remembered that the ordinary change of trypsinogen to trypsin is autocatalytic because the reaction is catalyzed by trypsin and the end product of the reaction is more trypsin. Hence the amount of catalyst in the system is continually increasing. Enterokinase acts best on trypsinogen at pH 6 to 9 where the autocatalytic activation also occurs most rapidly. The autocatalytic reaction rate, however, is proportional to the amount of trypsinogen. Hence by using very dilute trypsinogen solutions the autocatalytic rate may be made almost negligibly small. The autolysis of the trypsin formed, which also occurs best between pH 7 and 9, may be minimized by working at a temperature of 5°. The transformation of trypsinogen to inert protein also occurs best in the alkaline range, but this reaction may be

minimized by working at a pH near 6 where very little of the inert protein is formed.

Combining all these special requirements together it is possible to allow enterokinase to transform trypsinogen to trypsin under conditions where the interfering reactions play only a small part in the end result (44, 45). Then the reaction course was found to be monomolecular. The velocity constant was proportional to the concentration of enterokinase. The ultimate amount of trypsin formed was practically independent of the concentration of kinase. The behavior of enterokinase was thus shown to be like that of an enzyme catalyzing the change of trypsinogen to trypsin.

It is evident that while at pH 6 (and below) enterokinase acts almost like a typical enzyme, above pH 6 a large portion of the trypsinogen goes to inert protein. The lower the concentration of kinase the more inert protein is produced. Under such conditions the ultimate amount of trypsin produced would naturally be less as the amount of kinase used is less, giving the appearance that the kinase had combined with trypsinogen to form the active enzyme. Since these are the experimental conditions under which a great deal of earlier work was done it is not surprising that the latter conclusion was deduced from data so obtained. This knotty problem has been solved only after recognition of the fact that inert protein as well as active enzyme is formed during the activation of trypsinogen. It is small wonder that previous investigators, ignorant of this fact and without crystalline enzymes, went astray.

Bacterial proteinases.—Proteinases from microorganisms have for a long time been better known in industry than in the scientific laboratory. Recently, however, the proteolytic enzymes of some bacteria, mostly gas-producing anaerobes, have been studied, and what may turn out to be a new series of proteinases and peptidases with special characteristics has been found. To summarize and include also some earlier reports of the work of Maschmann (46, 47), four protein digesting enzymes have been distinguished:

(a) A proteinase fully active under aerobic conditions has been isolated from *B. pyocyaneus*, *B. prodigiosus*, and other organisms. It has a rather wide specificity, spreading from ovalbumin to peptone.

(b) A proteinase from gas-forming bacteria such as *B. perfringens*, *B. histolyticus*, and other anaerobes, which is fully active under aerobic conditions but exhibits a very narrow specificity. It digested only gelatin and gluten. This enzyme appears to be a typical extra-

cellular or secreted proteinase, for its concentration in the medium was observed to reach a maximum in a very short time (three or four hours) and without noticeable signs of cell autolysis. It appears also to be specially adapted to the splitting of collagen-like proteins, and furthermore seems typical of the gas-forming anaerobes. The effect of this secreted proteinase on the tissues in which the bacteria are resident is thought to have a connection with their ability to grow there, and possibly to their pathogenicity.

(c) Another proteinase from the anaerobes mentioned in (b) was found to be activated by sulphhydryl compounds and cyanide, but only toward certain substrates. It was not activated by ascorbic acid or ascorbic acid and iron, and appears to resemble the papainases. Maschmann calls this enzyme anaerobiase, and regards it definitely as an intracellular enzyme, common to many, perhaps all, anaerobes. Except for the splitting of gelatin or casein, the addition of a stable substance like a coenzyme was required. This situation may cast doubt on the unity of the enzyme, but preparations alike in this respect have been obtained from several species of bacteria.

(d) A proteinase from *B. sporogenes* that acts under aerobic conditions and resembles the proteinase of *B. pyocyaneus* except that it digested gelatin better than casein, and did not digest clupein, whereas the opposite was found for the pyocyaneus enzyme.

These bacterial enzymes have been prepared, generally from very young bouillon cultures, by selective precipitation with such reagents as methyl alcohol, acetone, or ammonium sulphate, and purified by dialysis. The preparations exhibit very considerable purification over the cell-free culture fluid from which they were obtained. The purification obtained for instance in the case of (b) amounted to a 2000-fold concentration of the enzyme; that in the case of (d) to a 140- to 350-fold concentration.

The use of filtrates from very young cultures is apparently necessary for the preparation of proteinases free from peptidase since the latter activity appears in the culture medium at a later stage of growth. By using the filtrates from older cultures and the same general precipitation methods Maschmann was able to obtain information (discussed later) with regard to these bacterial peptidases. Many puzzling variations have been reported in the behavior of the proteinases when obtained from different sources in respect to increased activation by iron in the presence of sulphhydryl compounds. It developed, however, that the activity of the proteinase was found not to be

affected by iron, but that of the peptidases is affected by iron in the presence of sulphhydryl groups. As in other instances, confusion of proteinase with subsequent peptidase activity has been easy.

Other studies on bacterial proteinases, principally from the *Clostridium* group, are reported by Weil, Kocholaty & Smith (48) from which it appears that such characteristics as pH optima, activation by sulphhydryl groups, etc., are common to the proteinases of a number of members of this genus.

It must be remembered that bacterial enzymes are prone to vary with the composition of the culture media, and discussion of whether the bacterial proteinases are adaptive or constitutive is quite to the point. A curious instance is reported by Pozerski & Guélin (49) from which it appears that the solubility of the enzyme may depend upon the medium. The gelatinase formed by a type of *B. perfringens* was observed to diffuse through the medium when the organism was cultured on a synthetic medium containing glucose but it did not diffuse when the same medium without glucose was used.

Peptide hydrolysis by proteinases: The specificity of proteinases.—

It is now well recognized that some proteinases even after purification by crystallization, are able to split certain peptides, though by no means all. This work, particularly in the hands of Bergmann and his co-workers, has followed the lines already reported in an earlier review (50). The kind of peptide split varies with the individual proteinase and therefore furnishes evidence concerning the type of peptide linkage toward which the enzyme in question is active.

Fruton & Bergmann (51) have found a number of peptides to be split by pepsin. Carbobenzoxy-*l*-glutamyl-*l*-tyrosine was split into carbobenzoxy-glutamic acid and tyrosine by hog pepsin that was repeatedly crystallized. The splitting proceeded quite rapidly at pH 4 but slowly at pH 1.8 or 2. Carbobenzoxy-*l*-tyrosyl-*l*-tyrosine and carbobenzoxy-*l*-glycyl-*l*-tyrosine were also split by pepsin, though but slowly. Most of the usual laboratory peptides were not attacked by pepsin. All the peptides so far found to be hydrolyzed contained either tyrosine or phenylalanine. It is evident that the action of pepsin (at least in so far as peptide-splitting is concerned) depends on the nature of both the amino acids at the peptide bond that is hydrolyzed. The most readily hydrolyzed substrate, carbobenzoxy-*l*-glutamyl-*l*-tyrosine has a free α -carboxyl and a free γ -carboxyl near the vulnerable bond. The substitution of the α -carboxyl did not prevent the hydrolysis although it was retarded, as illustrated by the slower split-

ting of carbobenzoxy-*l*-glutamyl-*l*-tyrosine amide. A similar effect was produced by substitution of a γ -carboxyl, for carbobenzoxy-*l*-glutamyl-*l*-phenyl-alanine was also hydrolyzed slowly. Substitution of both carboxyls prevented the enzyme from splitting the substrate at all, as shown by the observation that carbobenzoxy-*l*-glutamyl-*l*-tyrosine amide was not split. It should be noted that it is not necessary for the peptide to end in tyrosine.

The sequence of the amino acids in the peptide as well as their nature seemed to affect the rate of hydrolysis with pepsin. For instance, carbobenzoxy-*l*-phenylalanyl-*l*-glutamic acid was found to split slowly, whereas carbobenzoxy-*l*-glutamyl-*l*-phenylalanine split faster. Incidentally, carbobenzoxy-*l*-glutamyl-*d*-phenylalanine was not split at all, showing that the enzyme possesses some form of optical specificity.

These experiments prove that pepsin can split linkages in peptides. They explain the well known fact that tyrosine appears as the free amino acid among the products of the peptic digestion of proteins. Pepsin is thus evidently not restricted to the splitting of peptide bonds in the central part of a peptide chain but may on occasion split such bonds at the end of a chain. Just how far this type of reaction takes place with the proteins themselves is, of course, not now evident. Since proteins may be degraded to peptides, pepsin may produce free amino acids from the latter.

Evidence that trypsin liberates free cystine from casein (as pepsin liberates tyrosine) is found in the work of Jones & Gersdorff (52). Cystine is readily liberated in alkaline solution but is destroyed thereafter. In nearly neutral digests it is liberated more slowly, but remains.

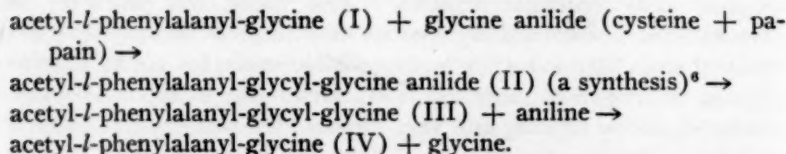
Bergmann, Fruton & Pollok (53) investigated the specificity of trypsin, using synthetic peptides. They found that ammonia is formed from α -benzoxy-*l*-arginine amide with great rapidity by crystalline trypsin. The substrate is also split by papain but not by chymotrypsin. Hofmann & Bergmann (54) found that α -hippuryl-*l*-lysine amide is split by trypsin, also very rapidly. On this account there is no need to assume the existence of the previously reported heterotrypsin as a separate proteolytic enzyme.

Earlier work of Bergmann's has clearly shown differences between proteinases in their behavior toward the same peptide, as reported in a previous review (50). Thus, with benzoyl-*l*-tyrosyl-glycine amide, chymotrypsin split off benzoyl-tyrosine, papain split off ammonia, and

trypsin had no action. The production of ammonia from peptide and amino acid amides may not necessarily be a reaction akin to the splitting of a peptide bond, but it is nevertheless a genuine reaction of some proteinases. Ammonia was formed from benzoyl-arginine amide by crystalline trypsin (53) and from hippuryl amide by crystalline papain (36).

Papain not only liberates ammonia from a number of synthetic substrates, but was observed by Damodaran & Ananta-Narayanan (55) to do so from protein digests. Neither pepsin nor trypsin acted thus. From the experiments with synthetic peptides one would not expect pepsin or chymotrypsin to act as deamidating enzymes, for the peptides they split are usually of decidedly acid character. Evidently the tryptic digestion of casein, edestin, and gliadin (the proteins used) does not produce amide-bearing fragments suitable for deamidation.

Hemolysis by trypsin was found to be accelerated by a heat stable factor in embryo tissue (56). A surprising effect of horse serum on the splitting of glycine anilide or glutamic acid monoanilide with papain and cysteine was observed by Behrens & Bergmann (57). These anilides are entirely resistant to papain under ordinary conditions, but are hydrolyzed when horse serum is present in the system. Moreover, it was found that the addition of acetyl-*dl*-phenylalanine, acetyl-*l*-phenylalanyl-glycine, or carbobenzoxy-phenylalanyl-glycine produced the same effect as the horse serum. The use of a synthetic substance gave an opportunity to investigate the reaction, and the reason for the splitting of these otherwise inert anilides is now clear. It is due to a series of reactions in which the acetylphenylalanine takes part, beginning with a synthesis catalyzed by the papain. The reactions, as worked out by Behrens & Bergmann, are as follows:



(IV) may recombine with the aniline formed from (II), giving acetyl-*l*-phenylalanyl-glycine anilide, or the whole process could repeat itself,

* A number of such syntheses of anilides, carried out with papain, bromelin and hog-liver cathepsin, have been described in earlier publications from the same laboratory (58, 59).

splitting more of the glycine anilide. The essential steps in this series of reactions have been demonstrated by the isolation of the intermediates in satisfactory yield from this and similar systems. Control experiments were possible because inactive papain (without cysteine) did not act in the manner described, nor was acetyl-*D*-phenylalanyl-glycine able to replace the *L*- form.

It is evident from this that the fate of a substance undergoing enzymic hydrolysis may differ, depending on the presence or absence of other substances that act as "co-substrates." Something of this sort may explain the old observation of Abderhalden & Ehrenwall (60) that peptidase activity is produced slowly in erepsin-free trypsin preparations on the addition of small quantities of several substances containing free hydroxy or amino groups, notably by dihydroxy-phenylalanine.

A study of the protein-digesting system of beef spleen has been made by Fruton & Bergmann (61) using the crude enzyme preparation made by fractional precipitation with ammonium sulphate. The digestion of proteins themselves by the spleen preparation was not studied, but a long series of peptides was tested. The preparation hydrolyzed a considerable number of peptides containing tyrosine or phenylalanine and glutamic acid. One of these peptides, namely carbobenzoxy-*L*-glutamyl-*L*-tyrosine, was found to be split easily without the addition of any activator. On the other hand, glycyl-*L*-glutamyl-*L*-tyrosine, its carbobenzoxy derivative and some other peptides were split much better in the presence of cysteine than in its absence. A third group of peptides including benzoyl-arginine amide and carbobenzoxy-glycyl-glycine required the presence of sulphhydryl in order to be split at all, whereas a fourth set of peptides, including glycyl-glycine, leucyl-glycine, and leucyl-diglycine, were split in the presence of either cysteine or ascorbic acid. The ascorbic acid activation only took place with those substrates containing a free amino group. So far as these synthetic peptides are concerned there are thus at least three types of proteolytic activity in the spleen—that requiring no activator, that requiring sulphhydryl, and that requiring either sulphhydryl or ascorbic acid.

The authors have been careful to avoid the conclusion which a number of years ago would have been regarded as self-evident, namely that the spleen contained one or more peptidases in addition to the well-recognized proteinase that exists there. Since the Bergmann school has found that so many peptides are split by protein-splitting

enzymes, it becomes difficult to tell whether the splitting of a peptide is due to a peptidase or to a proteinase. A definite statement cannot be made in such an instance until the several enzymes present, if there are several, have been separated from each other. On the other hand, it seems to the reviewer that there is no reason to discard entirely the view that separate proteinases and peptidases are responsible. The fact that some peptide-splitting is activated by sulphhydryl whereas other is unaffected seems to point to two enzymes rather than to one, particularly since it is recognized that the catheptic proteinase itself (from liver) is not activated by sulphhydryl, nor inhibited by iodoacetate.

Experiments on peptidases.—The peptidase picture has always been a puzzling one without the added complication that true proteinases may also split peptides. Except carboxypeptidase, which was crystallized by Anson, none of the peptidases has been obtained in a state of proved purity. Yet outstanding recent contributions to peptidase chemistry indicate that peptidase action is extremely dependent upon the presence of substances that may not always occur with the enzyme. The existence of what Behrens & Bergmann term co-substrates (see previous discussion) is probably not confined to the action of papain. In principle this observation alone gives a possible explanation of many contradictory statements in the literature of peptidase action, for it is clear that the presence of a co-substrate may permit an otherwise impossible reaction, thus producing an apparent extension of the enzymic specificity.

The effect of metals on the activity of peptidases is a matter of no less importance, for Johnson, Maschmann, and others have clearly shown that the activity of a peptidase toward some peptides may be altered by the presence of magnesium, manganese, and other metals, the particular effect depending, among other things, on the metal used.

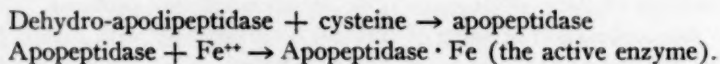
Thus the leucyl peptidase in hog erepsin (substrate leucyl-glycine and other leucyl peptides) was found to be activated by magnesium. Berger & Johnson (62), continuing Johnson's work, observed that manganese may be more effective than magnesium, and in addition activated diglycine hydrolysis, which magnesium does not. It is possible that manganese activates more than the one peptidase. Cobalt as well as manganese was an efficient activator of diglycine hydrolysis by malt peptidase, and zinc was observed to activate peptidase from several molds and yeasts in the splitting of diglycine. Microbial polypeptidases were also investigated. Some of these were found to be

activated by zinc salts but not by magnesium or by manganese. These were typical polypeptidases in that they hydrolyzed dipeptides very slowly, if at all. Leucyl peptidases were found in malt, cabbage, and spinach by Berger & Johnson (63). When prepared by acetone precipitation from the press juice, these peptidases appeared to be identical with the leucyl peptidase of the hog. The authors note that they found the malt peptidase to hydrolyze *D*-leucyl-glycine, although comparatively slowly. It is thus apparent to the general reader that the activation of the peptidases is by no means a finished problem.

An explanation of some of this puzzling behavior with metals has been advanced by Maschmann (47, 64) based on his extensive work with the peptidases of bacteria, particularly those that appear in solution in bouillon cultures of anaerobes, such as *botulinus* (A, B, C, and D), the Welch bacillus, *histolyticus*, and others. These enzymes have been named "anaeropeptidases" by Maschmann. The work has been confined largely to the study of dipeptide splitting. The cell-free bouillon cultures were very feebly, if at all, active toward simple peptides. They became so on the addition of cysteine with iron or manganese, but not with either cysteine or metal alone. Protein fractions removed from the culture fluid and further purified by dialysis provided a decided concentration and purification of the enzymes. It is noteworthy that cysteine and several other sulphur compounds with reducing properties (including glutathione, which was decidedly inferior in this respect) were found to act upon the enzyme protein quickly but nevertheless with a measurable speed. The subsequent addition of the metal formed the active enzyme at once; prior to the addition of the cysteine the metal had no effect. The splitting of leucine and glycine peptides was best with iron, that of alanine peptides was more favored by manganese. The iron-containing enzymes were totally inhibited by cyanide, though reactivated if the supply of iron was renewed. The manganese system on the other hand was not inhibited by cyanide, and the addition of manganese to a cyanide-inhibited iron preparation produced again the activity observed before inhibition. Iodoacetate had little effect on the active complexes, thus apparently eliminating a sulphhydryl group as the active center of the peptidase.

Maschmann suggests that his data show the existence of inactive enzyme proteins (apoenzymes) that combine with various metals to form peptidases with somewhat differing properties. The protein of the culture fluid must first be altered by the cysteine before it may

combine with the metal. The effect of cysteine, thioglycollic acid, and the like is attributed to a reducing action; that of cyanide on the iron complex is explained as removal of the metal. The bacterial dipeptidases are therefore thought to be metal proteids. Such compounds could hardly be expected to exist in the culture liquid, for the hydrogen sulphide nearly always present would decompose them. The necessity for both a reducing substance and a heavy metal has been explained as follows:



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CHEMISTRY OF THE CARBOHYDRATES AND GLYCOSIDES

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Since the chapters on carbohydrate chemistry in volumes VII and VIII of this *Review* were devoted largely to the polysaccharides, the present chapter will be confined to the sugars and glycosides. The literature for 1937, 1938, and 1939 has been considered, but completeness is not claimed, nor has any effort been made to arrange the subjects in the order of their importance. On account of limited space, it has been necessary to restrict the discussion to a few subjects only and to omit reference to many interesting contributions. As an aid in the correlation of data and in the hope of stimulating investigation, considerable freedom has been taken in the interpretation of experimental work, and in making generalizations.

When one surveys the recent developments in carbohydrate chemistry, one is impressed with the realization that the structures and configurations of the simple sugars and their derivatives have been proved, and that many investigators have turned their attention to the explanation of the differences among the various sugars and to the correlation of properties by extensive generalization.

In 1939, carbohydrate chemists were favored by the publication of an excellent book by Micheel (1) on the chemistry of the sugars and polysaccharides, and a report of progress in the chemistry of the carbohydrates by the same author (2).

STEREISOMERISM AND CHEMICAL PROPERTIES

Configuration of the pyranoid ring.—After many years of controversy, the ring structures of the methyl glycosides, as originally determined by the methylation method, have been accepted and the configurations of all of the asymmetric carbons have been established (3). It is becoming increasingly evident that the configurations of the atoms comprising the sugar ring influence in marked degree the reactions and properties of the sugars and their derivatives (4, 5, 6). In the pyranose series, the different configurations of the first carbon give rise to the α - and β -isomers, while the different configurations of carbons 2, 3, 4, and 5 give rise to the various sugars. These sugars may

be considered in groups according to whether the ring configuration corresponds to that of glucose, mannose, galactose, talose, gulose, idose, allose, or altrose (4). The pentoses differ from the hexoses in that the CH_2OH group is replaced by hydrogen, while the heptoses and higher sugars differ from the hexoses merely in the number and in the stereoisomeric arrangement of the atoms in the side chain. The resemblance of substances of like configuration appears to be more pronounced in the ring modifications than in the open chain modifications (7, 8). In an open chain compound exhibiting free rotation, no *cis-trans* relationship exists between the various groups, but when the atoms are joined in a ring, the restriction of free rotation gives rise to definite *cis-trans* relationships. *Cis-trans* relationships play an important part in many carbohydrate reactions, among which may be mentioned those with acetone (9), boric acid (10), lead tetraacetate (11), bromine (12), and even those with alkaline copper reagents (13). It will be brought out in the discussion of the Walden inversion that the *cis-trans* relationships influence the course of substitution reactions in a very remarkable manner.

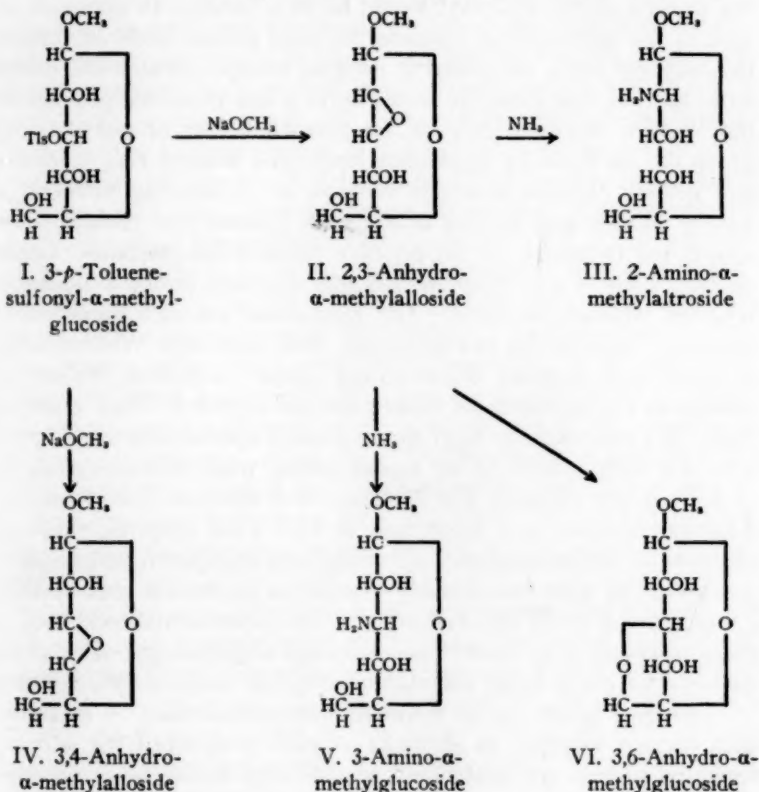
Walden inversion.—Despite many years of research, the conditions determining the occurrence and nonoccurrence of Walden inversion still remain one of the outstanding problems of organic chemistry. The steric course of any replacement reaction depends on its mechanism, and the predominance of one mechanism over another is influenced by both the reacting substances and the experimental conditions (14, 15, 16). Bartlett & Knox (17) prepared some bicyclic compounds in which Walden inversion is not possible and found that certain substitution reactions take place but that others are inhibited. Therefore, the reaction mechanisms may be divided into those which proceed with Walden inversion and those which proceed without inversion. In a Walden inversion, according to the hypothesis first suggested by Le Bel (18) and later amplified by Lewis (19), addition and dissociation take place simultaneously. The entering group approaches the carbon atom from the side opposite the group to be replaced and forms a carbon bond, while simultaneously, the group to be displaced departs and, by a slight shift of the kernel, the asymmetric carbon atom becomes the center of a new tetrahedron. Since this concept appears to explain and classify many diverse observations, the reviewer has taken the liberty of applying it freely to certain work in the carbohydrate field.

It has been established that the replacement of the *p*-toluenesul-

fonyl (tosyl) group by an acetyl or a hydroxyl group ordinarily leads to inversion (20). With sugar derivatives having a free hydroxyl available on a neighboring carbon atom, removal of the tosyl group usually results in the formation of an anhydro ring with inversion. In 1936, the formation and splitting of anhydro compounds were reviewed in this publication and numerous examples given (21), but activity in the field has continued and many new results of practical and theoretical importance have been obtained.

Formulas I to VI, based on the work of Peat & Wiggins (22) and Ohle & Wilcke (23), illustrate the production of anhydro derivatives by the alkaline replacement of the tosyl group, and the cleavage of the ring by the introduction of a negative group with inversion.

After extensive investigation, Ohle & Schultz (24) concluded that the formation of an ethylene oxide ring occurs only when the hy-



droxyl and sulfonyl groups on adjacent carbons are *trans*. A similar conclusion was reached by Müller, Móricz & Verner (25), who investigated certain methylsulfonyl derivatives and found that the ethylene oxide ring is formed only with Walden inversion and that sulfonyl derivatives which cannot form an anhydro ring with Walden inversion exhibit unusual stability toward alkaline hydrolysis. Bell & Williamson (26) had commented previously upon the extraordinary stability of 2-methyl-3,4-isopropylidene-6-*p*-toluenesulfonyl- α -methyl-*d*-galactoside toward alkaline hydrolysis. These observations are in complete harmony with the concept that the hydroxyl forming the anhydro ring approaches the face of the carbon on the opposite side from the tosyl group and combines, causing a Walden inversion. The formation of the ethylene oxide ring, rather than the butylene oxide ring, might be attributed to the fact that in forming the ethylene oxide the entering oxygen of the hydroxyl would be in a position to approach the face of the carbon atom opposite the tosyl group, while in forming the butylene oxide the entering oxygen would approach the carbon atom laterally and therefore would be in a less favorable position for the Walden inversion type of reaction. Whether or not the tosyl group can be removed by alkaline hydrolysis without ring formation and without Walden inversion remains an outstanding problem. It will be recalled that Bartlett & Knox (17) found that Walden inversion is not necessary for all negative replacement reactions. Consequently, there is no *a priori* reason why the tosyl group could not be replaced without inversion. The mechanism of such replacement, however, would differ fundamentally from that with Walden inversion. Several reactions which do not appear to involve Walden inversion or the formation of oxide rings are reported. Thus, Cutler & Peat (27) removed the tosyl group from 2-toluenesulfonyl-3,4,6-trimethyl- β -methylglucoside by saponification with alcoholic ammonia at 175° under pressure for 72 hours and obtained 3,4,6-trimethyl- β -methylglucoside, and Robertson & Gall (28) removed the tosyl group from 3-toluenesulfonyl-2,5-dimethyl- β -methylxylofuranoside by saponification with hot alcoholic potassium hydroxide and obtained 2,5-dimethyl- β -methylxylofuranoside. The experimental conditions of these reactions were severe and additional experimental work seems desirable before drawing conclusions as to the course of the reaction.

The tosyl group can be removed without inversion by treatment with sodium amalgam in alcoholic solution even when the adjacent hydroxyl groups are methylated so that ring formation is not pos-

sible (29). Instead of being a simple hydrolysis, the cleavage with sodium amalgam may be a reductive cleavage similar to the reductive cleavage of nitrates.

The opening of the anhydro ring with Walden inversion takes place in two directions, and under favorable conditions two derivatives may be isolated (30). For instance, in the 2,3-anhydro- α -methyl-*d*-alloside represented by formula II, the ethylene oxide ring may be broken with inversion at carbon 2 to give III or, at carbon 3 to give V. According to the concept of the Walden inversion previously outlined, the negative substituent group (NH_2) approaches carbon 2 (or carbon 3) from the side opposite the oxide ring and as the new bond is made with the carbon, the ring oxygen is released and is free to combine with a proton and form the hydroxyl which remains with the other carbon. By the use of suitable reagents, various negative groups may be introduced into the sugar molecule (31). Aqueous alkali introduces the hydroxyl group; ammonia the amino group; sodium methylate the methoxyl group; and hydrochloric acid the chloride group. Several papers relating to the cleavage of anhydro derivatives with ammonia are of particular interest (22, 32). The configuration of the biologically important chitosamine has been established as *d*-glucosamine by showing that its derivative, *N*-acetyl-trimethyl- β -methylglucosaminide, is identical with that prepared by the cleavage of 4,6-dimethyl-2,3-anhydro- β -methyl-*d*-mannoside with ammonia (33). The anhydro ring in 5,6-anhydro-1,2-acetone-*d*-glucofuranose is opened by treatment with alanine ester and after saponification and removal of the acetone, 6-*N*-alanino-*d*-glucose is obtained. This provides a method of combining amino acids with the carbohydrates to produce compounds of interest in relation to the proteins and enzymes (34). Treatment of 5,6-anhydro-acetone sugars with phenols in the presence of pyridine gives 6-phenyl ethers (35). The formation of 6-alkyl ethers by the addition of alcohols to 5,6-anhydro-acetone-*d*-glucose was investigated by Ohle & Tessmar (36) who found that the rate of reaction and the equilibrium constants depend on the nature of the alcohol.

Several products containing oxide rings other than the ethylene oxide ring have been reported. By treating 3-toluenesulfonyl-acetyl- β -methylglucopyranoside with sodium methylate, Peat & Wiggins (22) obtained in addition to 2,3- and 3,4-anhydro- β -methylalloside, 3,6-anhydro- β -methylglucoside. As proved by Ohle & Wilcke (23), the 3,6-anhydro- β -methylglucoside is formed from the 2,3-anhydro- β -

methylalloside, by what may be termed an intramolecular cleavage. (See formula VI.) Presumably, the hydroxyl on carbon 6 in the 2,3-anhydro- β -methylalloside approaches carbon 3 from the side opposite the ethylene oxide ring, thereby releasing the ring oxygen and forming a new ring on the opposite side of carbon 3.

There appears to be some uncertainty in regard to the products of the hydrolysis of tosyl derivatives which cannot form anhydro rings but which can form anhydrides. By treating 1-acetyl-2,3,6-trimethyl-4-toluenesulfonyl-*d*-glucopyranose and 1-acetyl-2,3,6-trimethyl-5-toluenesulfonyl-*d*-glucofuranose with sodium methylate, Hess & Heumann (37) obtained trimethyl hexose anhydrides, presumably with Walden inversion. On the other hand, by treating 3-toluenesulfonyl-2,4,6-trimethylgalactose with sodium methylate, Percival & Percival (38) obtained 2,4,6-trimethylgalactose in low yield.

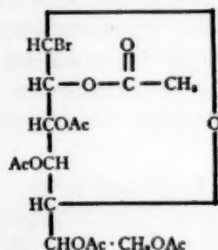
The cleavage of the anhydro derivatives with hydrogen chloride, in acetone or other suitable solvent, forms a convenient means for the preparation of chlorohydrins. Conversely, the chlorohydrins, on treatment with silver oxide, give anhydro derivatives (39).

Before leaving the subject of the anhydro sugars, it is of interest to note that 2,4-dimethyl-3,6-anhydro-methyl-*l*-galactoside was separated, apparently nearly simultaneously, by Hands & Peat (40) and by Percival, Somerville & Forbes (41) from methylated agar-agar, while its enantiomorph was synthesized by Haworth, Jackson & Smith (*see* 40), and by Percival & Forbes (42).

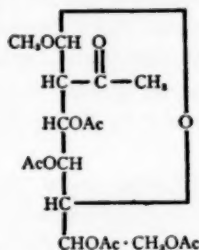
ESTERS AND RELATED SUBSTANCES

Acetohalogen sugars.—Application of the forementioned concept of the Walden inversion is of practical and theoretical importance in relation to the Koenigs-Knorr reaction in all of its modifications. Micheel & Micheel (43) showed by a number of examples that acetohalogen sugars having the halogen and adjacent acetyl group in the *cis* position yield quaternary ammonium salts with inversion, but that those having the halogen and adjacent acetyl group in the *trans* position do not give such compounds. Tipson (44) has pointed out that the normal acetates which have been obtained in the crystalline condition from the acetohalogen sugars and silver acetate are those having the acetyl groups of carbons 1 and 2 in the *trans* position. Pigman & Isbell (45) observed that orthomethylacetates may be formed from acetohalogen sugars in methyl alcoholic solution when the halogen and the acetyl groups of carbons 2 and 3 occupy *trans* positions. By se-

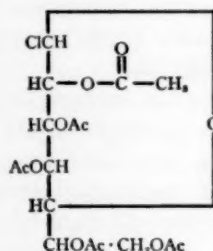
lecting the proper acetohalogen sugars and experimental conditions, either the normal glycosides or the orthoesters can be obtained. Thus, α -bromopentaacetyl-*d*- α -glucoheptose VII after treatment with methyl alcohol and silver oxide gives β -methylpentaacetyl-*d*- α -glucoheptoside VIII. β -Chloropentaacetyl-*d*- α -glucoheptose IX after similar treatment gives α -methylpentaacetyl-*d*- α -glucoheptoside X, but on treatment with methyl alcohol and quinoline, IX yields the orthoacetate XI (46). From an examination of the formulas, it may be ob-



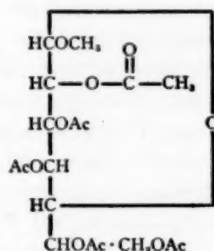
VII. α -Bromopentaacetyl-*d*- α -glucoheptose



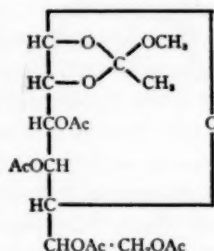
VIII. β -Methylpentaacetyl-*d*- α -glucoheptoside



IX. β -Chloropentaacetyl-*d*- α -glucoheptose



X. α -Methylpentaacetyl-*d*- α -glucoheptoside



XI. Tetraacetyl-*d*- α -glucoheptose-1,2-ortho-methyl acetate

served that if the halogen atom and adjacent acetyl group are on the same side of the sugar ring (*cis*), the acetyl group cannot approach the side of carbon 1 opposite the halogen and hence replacement of the halogen atom must take place with a group from outside the molecule (extramolecular reaction). But, if the acetyl group on the adjacent carbon lies on the opposite side of the ring (*trans*), the acetyl group can approach the side of carbon 1 opposite the halogen and hence the

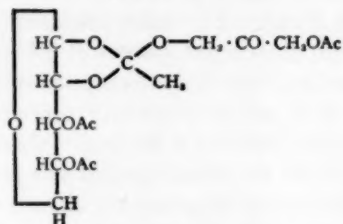
halogen can be replaced by an intramolecular orthoester reaction, which involves combination of the acetyl with carbon 1 and the simultaneous departure of the negative halide, accompanied by the addition of a methoxyl group to the acetyl carbon. The orthoester reactions appear to be accompanied by side reactions. Thus, treatment of *trans* acetobromo sugars with methyl alcohol and silver oxide gives the acetylated α - and β -methyl pyranosides in addition to the 1,2-orthomethyl ester (47). The formation of the β -methyl glycoside might be explained by an extramolecular reaction proceeding simultaneously with the orthoester reaction. The formation of the α -methyl glycosides from the α -halogen acetate seems unusual, but it might be explained by the addition of the methoxyl group to carbon 1 of the intermediate substance formed in the orthoester reaction, rather than to the acetyl carbon.

In the ketose series, the acetyl group on the adjacent CH_2OAc group can also approach the side of the glycosidic carbon opposite the halogen and give an orthoester. This may account for the orthoesters of fructose, turanose, and sorbose (48). A *trans* acetohalogen ketose like α -acetobromo-*d*-mannoheptulose might be expected to give a 1,2 or a 2,3-orthomethyl ester. However, Montgomery & Hudson (49) have reported the formation of α -methyl-pentaacetyl-*d*-mannoheptulose from α -acetobromo-*d*-mannoheptulose. Whether or not an orthoacetate is formed also must await further investigation.

An intramolecular orthoester reaction may be the explanation of the difference in the behavior of *cis* and *trans* acetohalogen sugars on treatment with tertiary amines. In contrast to *trans* acetohalogen sugars, certain *trans* methylhalogen sugars yield quaternary ammonium salts. Thus, Hess & Heumann (50) found that β -chloro-5-benzoyl-2,3,6-trimethyl-*d*-glucofuranose with trimethylamine forms a quaternary ammonium salt. They considered this to be a deviation from the Micheel & Micheel rule (43), but since the compound carries a methyl group on carbon 2, rather than an acetyl group, the reaction is not entirely comparable with those cited by Micheel & Micheel.

The importance of the intramolecular orthoester reaction in the synthesis of disaccharides is borne out by the interesting orthoester derivatives of ribose prepared by Klingensmith & Evans (51) from dihydroxyacetone monoacetate and acetobromo-*d*- and *l*-ribose. The resulting crystalline 3,4-diacetylribose-1,2-ortho-3'-acetoxycetonyl acetate (XII) is the first known example of an orthoester carbohydrate derivative which contains a group other than methyl or ethyl

combined in the orthoester structure. In contrast to the methyl-orthoesters, these new orthoesters are susceptible to alkaline hydrolysis, perhaps by virtue of rearrangement to the enediol structure.



XII. 3,4-Diacetylribose-1,2-ortho-3'-acetoxyacetyl acetate

Cleavage of ester linkages.—By the use of water containing the heavy oxygen isotope, it has been shown that during the alkaline hydrolysis of carboxylic esters (52), as well as during acid hydrolysis (53), the break in the linkage occurs between the $\text{RC}-$ group and



the $-\text{OR}$ group. In the reverse reaction, ester formation, the $-\text{OR}$ group from the alcohol replaces the hydroxyl of the acid (54). A similar study with trimethyl phosphate (55) showed that in the cleavage of the phosphoric esters the $-\text{OR}$ group is removed also. This accounts for the absence of Walden inversion in the hydrolysis of acetates, benzoates, and phosphates. Unfortunately, the heavy oxygen method has not been applied to other esters, but the mechanism of the hydrolysis of tosyl, mesyl, and nitrate groups appears to be fundamentally different (14, 56) from that of the acetates and phosphates.

Mesyl (methylsulfonyl) derivatives.—The methylsulfonyl derivatives (mesyl) recently introduced to the carbohydrate field by Helferich & Gnüchtel (57) appear especially noteworthy. They have many of the unique properties of the *p*-toluenesulfonyl and benzenesulfonyl derivatives, yet the group is smaller and more readily soluble in water solution. Primary hydroxyl groups are esterified by the mesyl chloride more rapidly than secondary, but the introduction of several mesyl groups presents no difficulties. The mesyl esters of primary hydroxyl groups resemble the tosyl and nitrate compounds in that they react with sodium iodide to give the corresponding iodo

compounds; additionally, mesyl esters of secondary hydroxyl groups may be replaced by iodine. Curiously, the mesyl compounds, when heated on a copper wire, impart a green color to a gas flame; in this respect, they resemble the halogen compounds.

Nitrates.—Bell & Synge (58) have prepared a number of partially methylated and esterified derivatives of glucose and have noted several unique reactions. 4,6-Ethylidene- β -methylglucoside-2,3-dinitrate when treated with acetic anhydride containing 0.1 per cent sulfuric acid gives 6-acetyl-4- α -acetoxyethyl- β -methylglucoside-2,3-dinitrate; apparently, the ethylidene group is opened by acetolysis. Treatment of the same 2,3-dinitrate with sodium iodide removes only one of the nitrate groups and gives 4,6-ethylidene- β -methylglucoside-3-nitrate [see reference to Oldham's work in (58)]. The nitrates are particularly suitable for preparing partially substituted sugar derivatives.

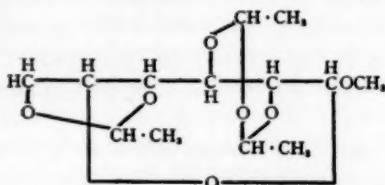
Phosphates.—In 1938, the chemistry and metabolism of the compounds of phosphorus, including the carbohydrate derivatives, were reviewed in this publication (59). Because of their importance in relation to the synthesis of glycogen, mention should be made of the preparation of 1-glucosyl, 1-mannosyl, and 1-galactosyl phosphates (60, 61) from the corresponding acetobromo sugars and silver phosphate, and of the preparation of pure 1-glucosyl phosphate from glycogen (62). Sugar phosphates are obtained in good yield by condensation of the acetohalogen sugars with silver dibenzyl phosphate, followed by removal of the benzyl groups by hydrogenation (63). Levene & Christman (64) synthesized 5-phospho-*d*-arabinose by the phosphorus oxychloride method. Cori, Schmidt & Cori synthesized a polysaccharide, similar in properties to glycogen, from 1-glucosyl phosphate in the presence of adenylic acid and an enzyme from muscle extract (65, 66, 67). 1-Glucosyl phosphate was converted by treatment with an enzyme from muscle extract to 6-glucosyl phosphate, while 1-mannosyl and 1-galactosyl phosphates were not changed (60). The migration in acid solution of the phosphate group from the secondary to the primary alcoholic groups and the reverse reaction have been established for the α - and β -glycerol phosphoric esters and the velocity constants determined (68).

Carbonates.—Haworth, Porter & Waite (69) noted some reactions of the sugar carbonates. *d*-Galactose and gaseous phosgene in dry acetone give diacetone-6-(chloroformyl)-*d*-galactose. *d*-Xylose under similar conditions gives 1,2-acetone-*d*-xylose-3,5-carbonate,

which on treatment with methyl alcohol, gives 5-carbomethoxyacetone-*d*-xylose. Treatment of *d*-mannofuranose dicarbonate with thionyl chloride gives α -chloro-*d*-mannose dicarbonate from which methyl-*d*-mannofuranoside dicarbonate may be readily prepared.

Chromatographic separations.—Azobenzene-*p*-benzoyl sugar derivatives of pronounced color and high molecular weight have been developed for use in chromatographic separations and micromanipulations (70, 71).

Ethylidene compounds.—Isopropylidene compounds form most readily on adjacent *cis* hydroxyl groups, while benzylidene compounds form most readily on alternate *cis* hydroxyls (9). Appel & Haworth (72) have found that paraldehyde condenses with α -methyl glucoside to give 2,3-oxidodiethylidene-4,6-ethylidene- α -methylglucoside (XIII). This compound is unique in that it contains a seven-membered ring formed from *trans* hydroxyl groups.



XIII. 2,3-Oxidodiethylidene-4,6-ethylidene- α -methylglucoside

INTERCONVERSION REACTIONS

Sugars in solution.—The existence of α - and β -pyranose modifications in solutions of the reducing sugars at equilibrium has been considered established for many years, but even today relatively little is known concerning the relative concentrations of furanose and open chain modifications when equilibrium is reached. The absorption spectra (73) indicate that solutions of the aldoses do not contain appreciable quantities of the aldehydo modifications. Solutions of levulose and of sorbose, however, show faint absorption at 2780 to 2800 Å, which is indicative of the open chain modification. The low intensity of the absorption band shows that the concentration of the open chain modification is low. This is in harmony with the chemical properties, which give little evidence of the presence of substantial quantities of open chain modifications.

Inasmuch as carbons 1 to 5 in the aldoses, and carbons 2 to 6 in

the ketoses, are primarily concerned in the formation of the ring isomers, the structures and configurations of these carbons determine in large measure the composition of the sugar solutions (4). If the aldoses are grouped according to the configurations of the first five carbons, it will be observed that the members in each group establish similar equilibrium states. However, the equilibrium state of an aldose differs greatly from that of a configurationally related ketose. This fact is not surprising since aldoses and ketoses differ in respect to the carbon atoms which are responsible for the α - and β -modifications and which form the ring isomers. Purves & Hudson (74) found that β -methyl- and β -benzyl-*d*-fructopyranosides are hydrolyzed by hydrochloric acid at a rate comparable with that of sucrose, a fructofuranoside, and pointed out that glycosides of the keto sugar, fructose, differ from those of the aldoses in that the furanoid and pyranoid ring types of fructose possess nearly the same stability toward acid hydrolysis. In this connection, it is of interest to note that the aldoses and aldonic acids also differ on the first carbon and that at equilibrium the six-membered pyranoid ring predominates, whereas the five-membered lactone ring predominates in the sugar acids.

The mutarotations in the ketose series show that the equilibrium solutions in some cases contain substantial proportions of the furanose modifications. From a comparison of the mutarotations of levulose and lactulose with the mutarotation of the fructofuranose set free from sucrose by invertase Isbell & Pigman (75) concluded that the mutarotation of levulose consists for the most part of a pyranose-furanose change, while that of lactulose consists of a furanose-pyranose change. Hence, crystalline lactulose appears to be a furanose. Isbell & Pigman's results do not preclude the presence of small quantities of the open chain and other modifications. The presence of such modifications may account for the small mutarotation of 3,4,6-trimethylfructose (75) and for the peculiarities of the mutarotation of tetramethyl- α -*d*-galactopyranose noted by Hendricks & Rundle (76).

Contrary to older observations, *l*-sorbose (77) and *d*-tagatose (78) exhibit small complex mutarotations, requiring the participation of at least three substances in the mutarotation reactions. The existence of several modifications of *l*-sorbose in solution is supported by the preparation of pyranose and furanose acetone derivatives (80). The initial and final solubilities of crystalline *l*-sorbose, however, indicate

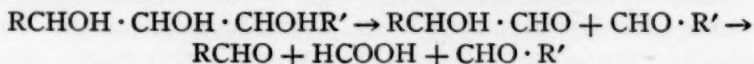
that at equilibrium the solution contains largely the modification known in the crystalline state which appears to be a pyranoside (79). The tendency of *l*-sorbitose to exist in solution as the α -modification (81) finds a counterpart in the greater stability of α -methyl-*l*-sorbitopyranoside toward hydrolysis. According to Schlubach & Graefe (82), β -methyl-*l*-sorbitopyranoside is hydrolyzed about 63 times as rapidly as the corresponding α -isomer.

In the aldose series, the mutarotations of α - and β -*d*-glucose, α - and β -*d*-mannose, α - and β -*d*-lyxose, α -*d*-gulose, and α -*d*-xylose follow within experimental error the first order equation. On the other hand, the mutarotations of α - and β -*d*-galactose (12, 83, 84), α - and β -*l*-arabinose (85), α - and β -*d*-talose (45), desoxygalactose (86), α -*d*-ribose (87), and those heptoses which have the galactose, talose, and idose structures (4) do not follow the first order equation and give unmistakable evidence of the presence of more than two isomers in their solutions when equilibrium is reached. The complex mutarotations may involve several reactions and products, but two reactions appear to predominate. The more rapid reaction involving considerable energy change appears to consist of an interconversion of the pyranose and furanose modifications, while the other reaction appears to be an interconversion of the α - and β -pyranose modifications. The reactions differ in their heats of activation and in their relative sensitivity to acid and basic catalysts (75).

Considerable attention has been given to the theory of the mutarotation mechanism and a number of papers have been published concerning the catalytic effect of acids and bases (88). Kuhn & Birkofer (89) studied the mutarotation of glucosides of secondary amines. They concluded that these glucosides add water to form quaternary ammonium bases, the cations of which undergo rearrangement through the intermediate open-chain Schiff base. This is somewhat analogous to the oxocyclic desmotropism frequently postulated as the mechanism of mutarotation. However, in the reviewer's opinion, it seems probable that the pyranose-furanose interconversion does not take place exclusively through the open chain modification. The conversion of the pyranose to the furanose modification by the simultaneous formation of a new ring and the rupture of the old would not seem inconsistent with the present concept of the Walden inversion. In this connection, it might be noted that pyranoses which give the complex mutarotations of the galactose type have opposite configurations of carbons 4 and 5.

OXIDATIVE CLEAVAGE OF THE CARBON CHAIN

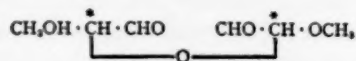
Cleavage of glycols.—Many applications of periodic acid and of lead tetraacetate oxidation in the cleavage of the carbon chain at glycol groups have established the general utility of these reagents in the preparation of lower sugars and sugar acids, and in the determination of configuration and structure. Therefore, a short résumé of the characteristics of the reactions seems opportune even though the subject was considered briefly in volume VI of this *Review* (p. 106). In contrast to most reactions involving the degradation of the carbohydrate molecule, the oxidative cleavage of glycol groups with either periodic acid (90, 91) or lead tetraacetate (92) takes place quantitatively at room temperature. A glycol group containing a primary hydroxyl yields formaldehyde which provides a means for the determination of such groups (93). In case the substance under examination contains only two adjacent secondary hydroxyl groups, the carbons carrying these hydroxyls are oxidized to aldehyde groups with the cleavage of the C-C bond. This requires one mole of the oxidant. If the substance contains three hydroxyls on adjoining carbons, it reacts with two moles of the oxidant in the following manner:



The structures of many substances may be determined from the quantitative relationship between the amount of periodic acid consumed and the number of adjacent free hydroxyl groups. For example, unsubstituted methyl pyranosides have three adjacent hydroxyls and consume two moles of the periodic acid, while methyl furanosides have two adjacent hydroxyls and consume only one mole (3). A more complex example of the determination of structure from the amount of periodic acid required for oxidation is found in the work of Hann, Maclay & Hudson (94) on α -diacetone dulcitol.

The periodic acid oxidation has proved to be exceedingly useful in the determination of the configuration of the glycosidic carbon in the methyl glycosides. In 1934, Hérrissey, Fleury & Joly (91) oxidized several methyl glycosides with periodic acid and suggested that the ring in the glycosides is broken between the central carbon atoms to give a dialdehyde in which the original glycosidic and ring-forming carbons are intact and united by an oxygen atom. Subsequently, Jackson & Hudson (3) proved that the methyl aldohexopyranosides give rise to four dialdehydes differing only in the configurations of

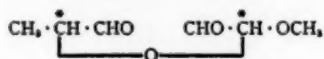
the two asymmetric carbon atoms, marked with asterisks in the following formula:



D'-methoxy-D-hydroxymethyl-
diglycolic aldehyde

The configurations of these carbons depend on the configurations of the glycosidic and ring-forming carbons in the parent glycoside. The methyl furanosides in the aldopentose series give the same products as the methyl aldohexopyranosides, and the methyl furanosides in the tetrose series give the same products as the methyl aldopentopyranosides (95). Since the several dialdehydes have widely different optical rotations, a measurement of the optical rotation after periodic acid oxidation shows which product is formed and thus establishes the configurations of the first and ring-forming carbons of the parent glycoside (3).

On periodic acid oxidation, α -methyl-*d*-isorhamnopyranoside gives rise to



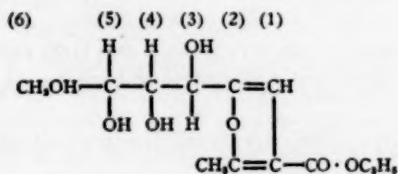
D'-methoxy-D-methyl-
diglycolic aldehyde

This product after further oxidation, and hydrolysis, gives oxalic and *d*-lactic acids. Maclay, Hann & Hudson pointed out that the *d*-lactic acid thus obtained (96) has the configuration which characterizes the *d*-sugar series.

McClenahan & Hockett (97) observed that the method for determining configuration as developed by Jackson & Hudson may be carried out with lead tetraacetate as well as with periodic acid. Until recently, lead tetraacetate had always been employed in nonaqueous solvents, e.g., benzene, nitrobenzene, chloroform, or glacial acetic acid, and emphasis was placed on the drying of such solvents. The exclusion of moisture was shown to be unnecessary by Baer, Grosheintz & Fischer (98), who conducted a number of oxidations in aqueous acetic acid. However, the reactions in aqueous and nonaqueous solvents differ in certain important respects. Thus, the oxidation of the methyl pyranosides with lead tetraacetate in aqueous solution re-

quires three moles of the oxidizing agent, instead of two, for the reason that, in the presence of water, the formic acid which is produced is oxidized to carbon dioxide (99).

Criegee, the originator of the lead tetraacetate method, noted that *cis* glycols are oxidized more readily than *trans* (93, 100). Later, Freudenberg & Rogers (101) made use of this difference in the reaction rates of *cis* and *trans* glycol groups to show that styracitol is a 1,5-anhydromannitol and that polygalitol is a 1,5-anhydrosorbitol. There appears to be a difference, also, in the reactivity of various glycol groups of polyhydroxy open chain compounds. Müller & Varga (102) have found that lead tetraacetate reacts preferentially with the 3,4-glycol group of methyl-tetrahydroxybutyl-furancarboxylic ethyl ester (XIV).



XIV. Methyl-tetrahydroxybutyl-furan-carboxylic ethyl ester

The tetrahydroxybutyl group is not capable of *cis-trans* isomerism and hence the directing force must arise from structural relations. Müller & Varga suggested that the double bond between carbons 1 and 2 weakens the 3,4-bond according to the principle of the Schmidt rule.

The periodic acid and the lead tetraacetate oxidations have been applied in the study of many products, among which may be mentioned: shikimic acid (103), saccharolactone (104), glucosaminic acid (105), 2,3,6-trimethyl- γ -galactonolactone (106), alginic acid (107), polygalacturonic acid (108), starch (109), dextrine (110), and trehalose (111).

Cleavage of enediols.—From a study of the oxidation of sugars with air in alkaline solution, Nef (112) concluded that under the influence of alkali a reducing sugar forms a 1,2-enediol which undergoes oxidation and cleavage to form formic acid and an aldonic acid. Spengler & Pfannenstiel (113) have shown that by using oxygen in place of air the cleavage of the sugar takes place smoothly and that aldonic acids are obtained in good yield. By the oxidation of perseu-

osones, hydroxyketonic acids, and other substances which contain enediol groups.

Ozonolysis.—Hurd & Filachione (120) demonstrated that aldehyde modifications of the lower sugars may be prepared by ozonolysis of the appropriate unsaturated benzoate. The benzoates are preferred to the acetates because of the susceptibility of the latter to hydrolysis.

Weerman reaction.—Haworth, Peat & Whetstone (121) investigated the oxidative degradation of the methylated sugar acid amides with sodium hypochlorite and found that the first stage involves the formation of an isocyanate, the subsequent transformation of which depends on the nature of the amide. The isocyanates from the α -hydroxy amides decompose with the liberation of sodium isocyanate, while the isocyanates from 2,3,4,6- and 2,3,5,6-tetramethyl-gluconamide yield cyclic urethans, which are readily converted to the lower sugar by dilute alkali.

SUGARS

Monosaccharides.—Mention of the production of lower sugars by the cleavage of substances containing glycol groups was made in connection with the lead tetraacetate oxidation. This method has been applied to the preparation of *l*-xylose (122), *d*-threose (123), and glyceraldehyde (124).

Inositol was prepared in 12 per cent yield from the precipitate obtained by adding lime to the steep water from the manufacture of corn starch (125). This method makes inositol available in large quantity. The reduction of sugars to polyhydroxyalcohols has become a very common laboratory procedure and several of the alcohols are now commercial products. The following sugars have been prepared from the corresponding alcohols by fermentation: dihydroxyacetone (126), *l*-erythrulose (127), *d*-xyloketose (128), *l*-psicose (129), per-seulose (130), and sorbose (131).

By condensing diazomethane with acetone-*d*-glyceroyl chloride, Iwaware (132) obtained a diazo ketone which on hydrolysis gave *d*-erythrulose. This new method should be applicable to the preparation of other ketoses. Iwaware & Kubota (133) gave detailed directions for the preparation of *l*-tagatose from pectic acid through the intermediate production of *l*-galactose and conversion by pyridine. By epimerization of *l*-galactonic acid, Fukunaga & Kubota (134) prepared *l*-talonic acid. They reduced *l*-talonic lactone to the sugar but

they could not bring the *l*-talose to crystallization. Glatthaar & Reichstein (135) prepared *l*-talose in similar manner, but failed to obtain crystals even in the course of three years. Both the α - and β -modifications of *d*-talose have been prepared in the crystalline state (45). As shown by Amadori (136), the condensation product of *p*-toluidine and glucose (*N-p*-tolyl-*d*-glucopyranoside) rearranges upon heating to form an isomer. Kuhn & Weygand (137) have found that this isomer is *N-p*-tolyl-*d*-isoglucosamine and that on hydrogenation it gives *N-p*-tolylmannamine. Presumably, the reaction takes place through an allylic rearrangement of the unknown Schiff base. Rearrangements of this character provide a new way of passing from *d*-glucose to *d*-fructose in the N-containing derivatives. Phelps (138) has worked out a very satisfactory method for the preparation of *d*-ribose in which yeast nucleic acid is hydrolyzed with magnesium hydroxide, and Brederick & Rothe (139) reported a method in which enzymatic hydrolysis of nucleic acid is employed. Whistler & Buchanan (140) investigated the preparation of β -*d*-glucose by Tanret's original method and gave detailed directions for the crystallization of the product. Pizzarello & Freudenburg (141) described a method for the preparation of *l*-galactonic acid by the oxidation of diacetone dulcitol by means of potassium permanganate. A number of higher methyloses have been prepared (142). Great confusion exists in regard to the nomenclature of the higher sugars (143), but, fortunately, a movement has been made toward clarification (144). Hudson (145) has shown that sedoheptulosan possesses an unusual structure, one of its rings apparently being an ethylene oxide and the other a septanoid. Richtmyer & Hudson (146) have obtained crystalline anhydro-altrose which by analogy should have the same structure as sedoheptulosan. As MacPhillamy & Elderfield (147) pointed out, digitalose might be 2-methyl-*l*-rhamnose, 2-methyl-*d*-fucose, 2-methyl-*d*-gulomethylose, or 2-methyl-*l*-altromethylose, but since they have synthesized 2-methyl-*l*-rhamnose and 2-methyl-*d*-fucose and have found them to be different from the natural sugar, digitalose must be either 2-methyl-*d*-gulomethylose or 2-methyl-*l*-altromethylose.

Disaccharides.—Zemplén and his co-workers, by condensation of 1-chloro-2,3,4-triacetylglucose with acetobromo sugars in the presence of mercuric acetate, obtained acetochloro derivatives of rutinose, 6- β -*l*-rhamnosido-*d*-glucose (148); primeverose, 6- β -*d*-xylosido-*d*-glucose (149); isoprimeverose, 6- α -*d*-xylosido-*d*-glucose (149); and by condensation of 1-chloro-2,3,4-triacetyl-*d*-galactose with 1-bromo-

2,3,4-triacetyl-*l*-rhamnose, they obtained an acetochloro derivative of robinobiose, 6- β -*l*-rhamnosido-*d*-galactose (148). Scillabiose was shown to be a *d*-glucosido-*l*-rhamnose (150) and hesperidin was shown to be hesperitin- β -rutinoside (151). Neohesperidin, recently isolated from bitter oranges, is a glycoside of a new biose, probably 4-*l*-rhamnosido-*d*-glucose (151). Reynolds & Evans (152) reported a very satisfactory method for the synthesis of gentiobiose from acetobromo-*d*-glucose and 1,2,3,4-tetraacetyl-*d*-glucose. Dauben & Evans (153) described the preparation of 6- β -*d*-glucosido-*d*-mannose from gentiobiose by the glycol method. Pacsu, Wilson & Graf (154) reported the preparation of 1- β -*d*-glucosido-*d*-fructose from acetobromo-*d*-glucose and 2,3,4,5-diacetone-*d*-fructose. Smith (155) isolated 3-*d*-galactosido-*l*-arabinose from the product obtained by the partial hydrolysis of arabic acid. Barry (156) presented evidence to show that the polysaccharide, laminarin, is built up of β -*d*-glucose units linked through the 1,3-positions. Isbell & Pigman (75) pointed out that the 5- α -*d*-glucosido-*d*-fructose structure for turanose is not correct and presented evidence to support the 3- α -*d*-glucosido-*d*-fructose structure proposed by them. Helferich & Pigman (157) found that lactulose, 4- β -*d*-galactosido-*d*-glucose, and neolactose, 4- β -*d*-galactosido-*d*-altrose, are hydrolyzed by sweet almond emulsin, while Richtmyer & Hudson (158) found that celtrobiose, 4- β -*d*-glucosido-*d*-altrose, is hydrolyzed likewise by sweet almond emulsin. These results confirm the β -glucosidic linkage in the above mentioned disaccharides. Tipson, Christman & Levene showed that the aldobionic acid from flaxseed mucilage is 2(*d*-galacturonopyranosido)-*l*-rhamnose (159). Levene and co-workers have investigated the structures of several oligosaccharides by means of catalytic reduction with hydrogen and have prepared a number of reference compounds to facilitate the identification of the methylated sugar alcohols (160). By the catalytic hydrogenation of substances containing carboxyl and uronic acid groups, it has been found that if the substance is esterified the carboxyl is reduced to an alcohol, but if the carboxyl is free, or in the form of a salt, it is not reduced; the aldehyde group is reduced if free, but if in the form of a glycoside, it is not reduced (161).

Reducing sugars were produced from sucrose and from starch by irradiation with visible and with ultraviolet light acting through uranyl salts as photosensitizers (162).

Sugar acids.—By the reduction of pentaacetyl gluconyl chloride, Cook & Major obtained aldehydogluco-pentaacetate, and by treatment

of the same substance with silver cyanide, they obtained pentaacetyl-2-keto-*d*-glucoheptonitrile, which was converted by hydrolysis to pentaacetyl-2-keto-*d*-glucoheptonic acid (163). The realization of possible uses of the acetylated acid chlorides stimulated investigation in this field (164).

Bernhauer & Knobloch (165) have ascertained that under proper conditions *Acetobacter suboxidans* acts on glucose in the presence of calcium carbonate to give a 37 per cent yield of 5-ketogluconate; the same organism acting on calcium gluconate gives 2-ketogluconic acid as the final product in about 75 per cent yield.

Sell & Link (166) gave detailed directions for the synthesis of galacturonic acid from α -*d*-galactose by the successive preparation of the following compounds: diacetone-*d*-galactose, potassium diacetone-*d*-galacturonate, diacetone-*d*-galacturonic acid, and crystalline galacturonic acid. Mottern & Cole (167) reported a method for the preparation of *d*-galacturonic acid in good yield from commercial pectin by the action of a commercial pectinase. Leutgoeb & Heinrich (168) described the preparation of *d*-glucuronic acid by the electrolytic oxidation of α -methyl *d*-glucoside. Hart & Everett (169) described a number of crystalline alkaloidal salts which should prove suitable for the identification of the keto acids.

Helferich & Peters (170) have developed a very satisfactory method for the production of ascorbic acids, in which aldoses and glyoxylic esters are condensed in alkaline solution by means of cyanides to yield ascorbic acids. Instead of aldoses, the acetylated cyanohydrins may be used. This synthesis is especially interesting because the carbon chain of the aldose is extended by two carbons in a new manner. Reichstein and his associates (171) have continued the study of the relationship between chemical constitution and antiscorbutic action in a series of ascorbic acid derivatives. Any essential alteration in the four atoms which form the ring in ascorbic acid destroys the activity; although a hydroxylated side chain appears to be essential, variations in this do not destroy the activity completely. The activity of 6-desoxy-*l*-ascorbic acid shows that a hydroxyl group on carbon 6 is not necessary for antiscorbutic action. By the action of sodium methylate on methylated 2-ketogluconic acids having a free hydroxyl group on carbon 5, Haworth, Hirst & Jones (172) obtained some derivatives containing six-membered rings and which may be regarded as analogues of ascorbic acid; they have properties resembling those of 3-methyl ascorbic acid. Evidence to show that the

hydroxyl of carbon 3 in *d*-arboascorbic acid is the most acid was obtained by Hawkins, Hirst & Jones (173), who found that *d*-arboascorbic acid gives a 3-methyl, and a 2,3-dimethyl ether with diazomethane.

The osones are of interest because of their use in the syntheses of ascorbic acids and because one of the tautomeric forms of the osones is closely related to the reductones and ascorbic acids (174). Several years ago, Evans, Nicoll, Strouse & Waring (175) found that osones are formed by the oxidation of sugars with copper acetate. By the use of methyl alcohol as a solvent, and carefully selected conditions, Weidenhagen (176) has improved the copper acetate method so as to obtain *l*-sorbosone and *l*-xylosone in approximately 60 per cent yield. Evans, Carr & Krantz (177) oxidized dihydroxyacetone with copper acetate and obtained the alcoholate of the trimer of hydroxypyruvic aldehyde in 87 per cent yield.

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THE CHEMISTRY OF THE ACYCLIC CONSTITUENTS¹ OF NATURAL FATS AND OILS

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THE FATTY ACIDS

In recent years one of the main advances in the chemistry of the fatty acids has been the preparation of the higher members of the series in chemically pure form with the result that it has been possible for accurate descriptions of their properties to be recorded. Such work has recently been continued by Francis & Piper who have determined the freezing, melting, and resolidifying points and crystal spacings of the pure normal C_{2n} fatty acids and their methyl and ethyl esters from C_{14} to C_{36} and C_{46} and for some members of the C_{2n-1} series from C_{17} to C_{29} . For symmetrical and unsymmetrical mixed triglycerides prepared synthetically from the pure acids similar data have also been recorded by Malkin *et al.* (11, 85). Palmitic and stearic acids have been obtained in a highly purified form by Guy & Smith. These authors have recorded the properties not only of the acids themselves but of several of their derivatives and of binary systems prepared from these derivatives.

Although the saturated fatty acids are by no means readily prepared in a pure state, the purification of the liquid unsaturated compounds presents even greater difficulties. These have been overcome to a great extent for oleic acid by Smith (104) who has prepared a sample of what is probably the purest specimen of this acid so far obtained. His procedure, for which the technical details are given, involved fractional distillation of methyl esters, followed by fractional crystallisation of the acids at -50° . The sample (m.p. $\alpha, 13.36 \pm 0.04^{\circ}$; $\beta, 16.25^{\circ}$), contained less than 0.2 per cent palmitic acid and 1.0 per cent stearic acid. Binary mixtures of saturated and unsaturated acids were also studied. A sample of relatively pure oleic acid has also been prepared by Hartsuch whose method of purification was based on similar principles.

The nature of linoleic acid which occurs so frequently in natural fats has for long been a subject of investigation, but the possibility

¹ The literature available up to November, 1939, has been examined.

of its unique importance was only discovered a few years ago when Burr & Burr showed it to be an essential accessory food factor for rats. The molecular structure of the acid has been discussed in various publications during the past twelve months. When the linoleic acid of seed fats is brominated it gives rise to both a crystalline (α) and a liquid (β) tetrabromo derivative. The plurality of the end-products suggests either that the original $\Delta 9:10 - 12:13$ —octadecadienoic acid exists in fats in different isomeric forms or that there is only one naturally occurring isomer which can be brominated or oxidised along different paths to give different derivatives or which can undergo partial change during these two processes. The problem was discussed some time ago by Green & Hilditch (32) and has now been re-examined from different points of view by various authors. Brown & Frankel have prepared α -linoleic acid by debromination of the crystalline tetrabromide and have also obtained the naturally occurring acid at least 93 per cent pure by low temperature fractional crystallisation of the unsaturated acids from corn oil. The fact that both preparations on bromination yield practically the same proportion of the crystalline tetrabromide, considered along with other properties, clearly indicates that the so-called α -linoleic acid (m.p. -6.8°) and the naturally occurring compound (m.p. -7°) are identical. In further papers by McCutcheon, starting with sunflower seed oil, and by Riemenschneider *et al.* using cotton seed oil, the β -linoleic acid has been studied in addition to the so-called " α " isomer. The general conclusion arrived at by these authors is that α -, β -, and natural linoleic acids are identical and consequently that only one linoleic acid is so far known to exist in seed fats. Diagrammatic explanations for the occurrence of the two bromo derivatives are given. Riemenschneider *et al.* base their conclusions largely on the fact that in their experiments both the natural and α -acids gave yields of crystalline bromo derivative amounting to about 45 per cent of theory, whereas the β -isomer, which was somewhat impure, gave a figure as high as 36.7 per cent. On the other hand, Hilditch & Jasperson (47) who were simultaneously studying the same problem obtained yields as high as 45 to 51 per cent for the tetrahydroxy- and crystalline tetrabromo derivatives from both the natural and α -acids but only such values as 18.2 and 23.9 per cent for the β -acid. Hilditch & Jasperson therefore concluded that the β -acid differed from the natural and α -acids, which were identical. They point out that since oleic and elaidic acid each give a bromo derivative which reverts to the original

acid on debromination, the 9:10 position probably does not take part in the formation of the β -linoleic acid, but that the latter acid results from a cis-trans isomerism taking place at the 12:13 linkage in a portion of the linoleic acid. On their hypothesis the so-called β -acid would consist of approximately equal parts of the original α - or natural acid and one which had undergone cis-trans isomerism at the 12:13 linkage. The preparation and study of elaidinised linoleic acid and its derivatives by Hilditch & Jasperson, reported in the same paper, and by Kass & Burr have also contributed considerably to the subject.

Brown & Frankel, in a paper already cited, reported that 0.906 gm. crystalline tetrabromide resulted from 1 gm. of pure linoleic acid, and therefore suggested that the linoleic acid content of a fat might be estimated approximately from the formula $1/90.6$ (weight of crystalline tetrabromide from 100 gm. of fatty acids) $\times 100$. Obviously such a formula would have to be well established before it could be applied to fats in general, for it is most probable that the proportion of crystalline bromide resulting from the octadecadienoic acid in a mixture may depend on several facts which are not at present thoroughly understood, such as the conditions of bromination and the nature of the other fatty acids present. Moreover, the method would clearly not be applicable to such fatty acid mixtures as those which are obtained from milk fat (33) and grass leaf fat (103), which yield no crystalline tetrabromo derivative and yet have been proved by oxidation methods to contain octadecadienoic acids. The nature of the linoleic acid in milk fat from cows fed on pasture has recently been the subject of a communication by Hilditch & Jasperson (48) but, although valuable ground has been explored, the precise nature of this particular octadecadienoic acid has not yet been elucidated.

The still more unsaturated linolenic acid, also widely distributed in certain vegetable fats, has been studied by Shinowara & Brown. A sample, 83 to 88 per cent pure, was obtained by low-temperature crystallisation of the fatty acids of linseed and perilla oils from acetone and light petroleum. A sample of the α -acid (m.p. -14.5°) prepared by debromination of the crystalline hexabromide did not appear to be identical with the natural product (m.p. -11.5°) since it gave a much lower yield of crystalline hexabromide. The authors suggest that linolenic acid might be estimated approximately from the yield of solid hexabromide by the method just discussed for linoleic acid, substituting 92 for 90.6 in the formula given above.

The separation of fatty acids from various mixtures by selective

adsorption on silica gel or alumina has been investigated by Kaufmann (62). The mixtures which he had studied include benzene solutions of myristic and stearic, lauric and stearic, oleic and linoleic, stearic, oleic and linoleic acids, and several others of a similar type. The mixed fatty acids of whale oil and linseed oil have also been tested. The results show that alumina selectively adsorbs the saturated acids, and that when only saturated acids are present it preferentially adsorbs those of higher molecular weight. Silica gel on the other hand tends to adsorb the lower constituents from trichlorethylene solutions of mixed saturated acids. Adsorption on silica gel as a step towards purifying fatty acids and glycerides has also been studied by Kurz, but he finds that the separation is not sufficiently sharp for preparative purposes. Magnesium sulphate has been used by Manunta for a similar process.

Further papers of importance which may be cited in connection with the fatty acids are those on the preparation of compounds containing deuterium by von Heyningen *et al.* and on the adsorption spectra of fatty acids by Miller *et al.*

The synthesis of glycerides and other fatty substances from fatty acids with the aid of trityl chloride or other derivative has been reviewed in detail by Verkade. Black & Overley have synthesised mono- and tri-linoleins using crystalline tetrabromostearic acid as their starting material, while such reactions as the production of mixed glycerides *in vitro* by heating an equimolecular mixture of tristearin and triolein have been described by Toyama.

VEGETABLE FATS

The majority of papers published each year on the chemical composition of the fats are concerned with those of vegetable origin, and since the period covered by the present review has proved no exception to the rule, it is only possible to deal very briefly with the large number of papers which have recently appeared on this particular section of the work.

The seed fat of *Neolitsea involucrata* has been examined by Gunde & Hilditch (35) who found that its component fatty acids contained as much as 86 per cent of lauric acid with 3 per cent of decanoic, four of myristic, four of oleic, and three of linoleic; 87 per cent of the glycerides were fully saturated and at least 66 per cent consisted of trilaurin. An analysis of the fruit coat fats showed that their fatty acids also contained lauric acid but only to the extent of 10

per cent, and this was accompanied by 28 per cent of palmitic, three of stearic, five of palmitoleic, 44 of oleic, and 10 of linoleic acid. The high content of lauric acid in the seed fat is characteristic of members of the Lauraceae family to which this plant belongs, but the fruit coat fat is somewhat unusual in containing so much of the C_{12} acid, for in most fruit coat fats so far examined the major acid components are palmitic, oleic, and linoleic. The same authors (36) have also analysed the fatty acids of *Salvadora oleoides* and *S. persica* and have found them to consist of about 1 per cent of decanoic, 20 of lauric, 54 of myristic, 20 of palmitic, and 5 of oleic acid. In the seed fat of *Irvingia gabonensis* (dika fat), workers from the same laboratories (9) have found 58.6 per cent of lauric acid and 33.4 of myristic with small amounts of C_{10} , C_{16} , and C_{18} acids, the chief component glyceride being dilauromyristin which was present to the extent of about 65 per cent. The corresponding fats of *I. barteria* and *I. elivieri* were much the same except that they contained some 40 per cent of lauric acid and 55 of myristic acid. High values of 31.6 and 14.7 per cent for the lauric and myristic acid contents of Dum palm nut fats have been obtained by Ubaldini, the other components being small amounts of octanoic, 1.3 per cent and decanoic acids, 2.7; and larger quantities of palmitic, 13.7; stearic, 4.7; and unsaturated acids, 30.5 per cent.

A particularly interesting type of fat has recently been discussed by Gunde & Hilditch (37) who have analysed the fatty acids obtained from the seed and fruit coat fats of *Celastrus paniculatus*, a deciduous shrub found in certain parts of Asia. By extracting a petroleum solution of the fat with 80 per cent aqueous methyl alcohol, a fraction was obtained containing no glycerides but consisting of esters in which formic, acetic, and benzoic acids were combined with a tetrahydroxy non-benzenoid compound for which the analytical data suggested a formula of the type $C_{14}H_{24}O_8$ or $C_{16}H_{26}O_8$. The acids obtained from the total seed fat contained 6.0 per cent of formic, 1.8 of acetic, 2.0 of benzoic, 20.1 of palmitic, 3.9 of stearic, 15.3 of oleic, 38.8 of linoleic, and 21.1 of linolenic acids. Those from the fruit coat were similar except that they contained less formic acid, 2.4 per cent; more acetic, 4.0; more oleic, 27.8; and less linoleic, 17.4 per cent.

A recent analysis of the oils from the white, red, and black mustards has been published by Dutt. The three oils appear to be very much alike both in their fatty acid content and in the small amounts of cyanides and isothiocyanates which they contain. The fatty acids

consist of 0.5 per cent each of myristic and stearic acids, 3.9 of behenic, 1.4 of lignoceric, 31.9 of oleic, 17.8 of linoleic, and 2.9 of linolenic acids with 42 per cent of erucic acid. Another particularly rich source of erucic acid is the seed fat of the annual nasturtium (*Tropaeolum var.*) which has been shown by Hilditch & Meara to contain about 40 per cent of trierucin. The fatty acids from the total fat consist of erucic acid, 82 per cent; oleic, 16; linoleic, 1; and saturated acids, 1 per cent, the greater part of the saturated acids being behenic. The trierucin (m.p. 31.5–32°) was separated and purified by crystallisation and its x-ray spectrum compared with that of the synthetic product. The high content of erucic acid, characteristic of the seeds of this species, makes them excellent sources of this particular fatty acid.

In connection with the occurrence of erucic acid in fats, a method for its detection in the presence of oleic acid has been published by Kaufmann & Fiedler (64). These authors have found that oleic acid breaks down to give lower oxidation products, very much more readily than erucic, and that the reaction conditions can easily be adjusted to cause erucic acid to form its dihydroxy derivative which is readily isolated while the oxidation of oleic acid proceeds further. Since as little as 2 per cent of rape oil in other vegetable oils was detected by this means, the method is likely to be of much value in detecting erucic acid in fats which contain it in only very small amounts. The same authors (65) found that the fatty acids obtained from Rocket seed oil (*Eruca sativa*) contain 58.8 per cent of erucic acid.

The oil of *Aleurites trisperma* is reported by Frahm & Koolhaas to contain 49 per cent of elaeostearic acid with 17 per cent of saturated acids, 11 of oleic, and 19 of linoleic acids.

Several seed fats which are rich in the commoner fatty acids such as oleic and linoleic have been analysed recently. *Asimona triloba* (Papaw) was found by Riebsomer *et al.* to yield 38 per cent of glycerides of which the fatty acids contained 59 per cent of oleic, 26 of linoleic, and small amounts of the following acids: palmitic, 2.3; stearic, 1.8; and arachidic, 1.5 per cent. According to Ueno & Ueda (123) Chinese tea seed yields an oil which is particularly rich in oleic acid (84 per cent) and which also contain small quantities of palmitic, stearic, and linoleic acids. In *Sterculia* oil the same authors found glycerides containing 45 per cent of oleic, 30 of linoleic, and 25 of palmitic and stearic acids, and have also shown that the liquid fatty acids of rice oil (124) consist of almost equal parts of oleic and lino-

leic acids. Grape seed oil from the Pfalz district of Germany has been analysed by Kaufmann & Sprick. The fatty acids consisted of saturated compounds (12 to 16 per cent), oleic acid (12 to 20 per cent), and linoleic acid (67 to 73 per cent). Another oil rich in unsaturated acids was obtained by Kaufmann & Fiedler (66) from pumpkin seed. It contained 55 per cent of linoleic acid and about 27 of oleic, 11 of palmitic, and 6 of stearic acids. Watermelon seed oil analysed by Nolte & von Loesecke also had a high content of linoleic acid, 63 per cent; the other fatty acids were oleic, 13; palmitic, 9; stearic, 6; and arachidic, 1 per cent.

Other vegetable seed and fruit oils for which recent analyses are available are ash seed oil (7), argan oil (16), Cycad and Nigaki oils (117), ben (Moringa) seed oil (59), oils of certain Formosan plants such as the pineapple, tomato, passiflora, and sugarapple (42), oils of the Cape gooseberry (78), of *Blepharis edulis* (96), of *Pongamia glabra*, Vent. (88), of *Oroxylum indicum*, Vent. (90), of cumaru (79), of akebi (72), of *Brachychiton diversifolium* (77), of *Quisqualis indica* (67), of *Stillingia sebifera*, Trillot (68), and of andiroba (2).

The fats isolated from the "germs," seed shells, and cotyledons of the seeds of *Theobroma cacao* Linn. have been analysed by Bauer & Seber. The "germs" were richest and the cotyledons poorest in linoleic acid, while the reverse was true for the saturated and oleic acids.

Two papers have appeared recently on the fatty constituents of flowers. Schmid & Hosse isolated the fat from dried corn-poppay flowers and found it to contain palmitic, stearic, oleic, and probably arachidic acids. By selective adsorption on alumina a compound or mixture of compounds with the mean molecular formula $C_{26}H_{58}OH$ was obtained from the unsaponifiable matter, while it is stated that the hydrocarbon $C_{27}H_{56}$ (m.p. 61.5°) was isolated and identified by x-ray analysis. The fat of the flowers of *Arnica montana* has been shown by Dieterle & Fay to yield 56 per cent of fatty acids of which half is oleic acid, the other components consisting mainly of palmitic acid with some hexanoic, octanoic, lauric, and a little stearic acid. An alcohol of high molecular weight, two sterols, and hydrocarbons were also found to be present.

Two examples of root fats have been analyzed recently. Manjunath & Rao (87) found the roots of *Bragantia wallachii*, Lour., to yield glycerides containing palmitic, lignoceric, oleic, and linoleic acids,

while the oil from *Kostus* root is reported by Ukita to contain palmitic acid together with two very unusual unsaturated products. For one of these products, an acid, he suggests the formula $C_{15}H_{22}O_3$ (m.p. 118.5°) and for the other, a lactone, he suggests $C_{15}H_{18}O_2$ (m.p. 60.5°). Further publications on the nature of such unusual compounds will certainly be awaited with interest.

In the literature of the past year a number of publications have appeared which refer to some relatively unusual fatty acids found in certain vegetable fats. For example, for recrystallised parinaric acid, a C_{18} acid from the fat of *Parinarium laurinum*, the spectral absorption curve has been obtained by Kaufmann *et al.* (63) and supports the formula $CH_3CH_2 \cdot (CH:CH)_4(CH_2)_7COOH$ previously put forward for this compound by Farmer & Sunderland. Another example is the seed fat from *Cleome viscosa* by Gupta & Dutt. In addition to myristic and palmitic acids, this fat yields an unusual compound which has been named "viscotic" acid, m.p. 97° , and apparent molecular formula $C_{27}H_{52}O_3$. On oxidation with dilute permanganate a dihydroxy derivative $C_{27}H_{54}O_5$ is formed. The seed fat of another Indian plant, *Santalum album*, Linn., has been studied by Manhuranath & Manjunath. The solid fatty acids isolated after saponification contained a small proportion of palmitic acid along with a solid unsaturated compound which has been named "santalbic acid." Its formula is given as $C_{18}H_{30}O_2$, m.p. $41-42^\circ$. It absorbs hydrogen to give stearic acid, but only liquid derivatives are obtained when it reacts with bromine or maleic anhydride. A study of the structure of this substance is now in progress. Gorlic, or dehydrochaulmoogric, acid prepared from chaulmoogra oil extracted from *Carpotroche brasiliensis* or *Oncoba echinata* has been studied further by Cole & Cardosa (14). Several properties of the acid (m.p. 6° ; $[\alpha]^{25}_D$, $+60.7^\circ$) and of its methyl and ethyl esters have been recorded. The same authors (15) have also isolated and studied four lower C_{2n} homologues of chaulmoogric acid.

Tsujimoto (116) has examined some unusual fatty acids which are combined as glycerides in Japan wax. When a petroleum solution of the wax was allowed to pass through Japanese Clay, a portion was preferentially adsorbed. On elution, it was found to contain a mixture of glycerides of which 24 per cent was composed of dibasic acids appearing to have the mean formula $C_{23}H_{44}O_4$.

Schuette & Vogel continuing their previous work on alfalfa seed oil have been investigating the small saturated acid fraction which it

contains. They have corrected a pre-existing impression that it consisted of the C_{17} acid, margaric, and have shown it to be a mixture of palmitic and stearic acids with possible traces of myristic and higher C_{2n} homologues.

A few years ago, hexadecenoic acid, the C_{16} analogue of oleic acid, would have been regarded as one of the more unusual fatty acids, at any rate as far as non-aquatic fats are concerned, but its presence in a great number of fats, not of aquatic origin, has recently been proved beyond dispute. Its distribution was recently reviewed by Fiedler and also by Hilditch (45), who shows that it is probably present in all natural fats, but that only those of aquatic or semi-aquatic origin contain it in more than very small amounts. That it seems to be present in many vegetable oils to the extent of less than 1 per cent has recently been discussed in detail by Hilditch & Jasper-son (49) who have also studied the C_{16} unsaturated acids in soybean oil (50). They found that the palmitoleic acid content of this particular oil was of the order of 0.5 per cent and proved that it had the double bond in the 9:10 position and was therefore identical with the palmitoleic acid from other vegetable and animal sources. The authors suggest that perhaps there is present also some hexadecadienoic acid, since the thiocyanogen values of their C_{16} ester fractions were always less than the iodine values, whereas a previous paper by Hilditch *et al.* (51) had shown that for $\Delta^{9:10}$ hexadecenoic acid the thiocyanogen and iodine values are equal. It may also be noted here that the presence of palmitoleic acid in fats of non-aquatic sources has been shown fairly recently by Longenecker for corn (80) and coconut oils (81), by Hilditch *et al.* for cow butter fat (52) and for the depot fat of the ox (53) and pig (51), and by Spadola & Riemenschneider for fats from such widely differing sources as the adipose tissue of rats, goat milk, and egg yolk.

Much progress has been made in recent years not only in elucidating the nature of the component fatty acids of vegetable fats, but also in determining the ways in which the glyceride molecules are constructed from these components. This work, undertaken mainly by Hilditch and his co-workers, is described from time to time in an important series of publications and has recently been the subject of a most interesting review (46). The three most recent publications of the series deal with the fatty acids and glycerides of (a) Borneo tallow (10), (b) dika fat, the seed fat of *Irvingia gabonensis* (9), and (c) the seed fat of a Malayan climbing plant, *Hodgsonia capniocarpa*

(55). The first of these is an example of a fat containing the three acids, palmitic, stearic, and oleic, as its principal components. These were found to be combined in the original fat as oleodistearins, 40 per cent; oleopalmitostearins, 31; steardioleins, 13; oleodipalmitins, 8; palmitodioleins, 3; and fully saturated glycerides, 5 per cent. The tendency for the glycerides to be as mixed as possible in their composition is very marked. The second example (dika fat) was chosen to give an example of a fat in which the two saturated acids, lauric and myristic, took the place of the more common palmitic and stearic in forming mixed glycerides with oleic acid. Actually, however, oleic acid was present in the particular sample of fat used to the extent of only 1.8 per cent, while the other fatty acids were decanoic, 3.0 per cent; lauric, 58.6; myristic, 33.4; palmitic, 2.0; and stearic, 1.1 per cent. By systematic crystallisation from acetone, the chief component glyceride was found to be dilauromyristin, 65 per cent, the other principal triglycerides being laurodimyristin, 13.5, and decolauro-myristin, 15.0. The third example, the seed fat of *Hodgsonia capniocarpa*, was of particular interest, in that it contained 24.3 per cent of linoleic acid in addition to the major components palmitic acid, 36.1 per cent; stearic, 9.5; and oleic, 26.5 per cent. It is, therefore, an instance of glyceride structure where the major components are two saturated and two unsaturated acids. The results in molar percentages showed that the original fat contained less than 3 per cent of fully saturated glycerides of which the main part was tripalmitin, the unsaturated glycerides being "oleo-" dipalmitins, 33 per cent; "oleo-" palmitostearins, 27; palmito- "dioleins," 24; and oleolinoleins, 13 per cent. The authors believe that in this instance the "oleo" glycerides would probably contain oleic and linoleic acids in approximately equal amounts. It is of importance to observe that although the saturated acids in the fat (51 per cent of the whole) very slightly exceeded the unsaturated acids, there was less than 3 per cent of fully saturated glycerides and as much as 13 per cent of molecules containing only unsaturated acids. In shea butter (34) where there is a higher proportion of unsaturated acids (53.7 per cent) but very much less linoleic (3.9 per cent) the content of tri-unsaturated glycerides was only 4.5 per cent.

ANIMAL FATS

There have been very few examples of the analysis of fat from land animals during the past year. One paper by Hilditch *et al.* (51)

deals with the influence of different planes of nutrition on the composition and synthesis of fat in the pig, and for this purpose various samples of fat have been examined and the analytical data recorded. The composition of the fats from various parts of the Virginia white-tailed deer have been analysed by Treadwell & Eckstein. The perirenal, pericardial, omental, pericaecal, and mesenteric fats all seem to be very similar as far as the saponification, thiocyanogen, and iodine values are concerned. These averaged 193.5, 27.2, and 30.4 respectively. From the thiocyanogen and iodine values and from lead soap separations of the solid acids the proportions of oleic, linoleic, and solid acids present were estimated. The mammary gland fat appeared to be somewhat more unsaturated than the others, for although it had almost the same saponification value, the thiocyanogen and iodine values were 38.9 and 40.6 respectively. The fatty acids from the glycerides of male goats are reported by Dhingra & Sharma (19) to consist of lauric, 3.5 per cent; myristic, 2.1; palmitic, 25.5; stearic, 28.1; arachidic, 2.4; and oleic acid, 38.4 per cent.

With regard to the analysis of animal fat, Hilditch & Pedelty (56) have described a very useful method for estimating, from various properties of the fatty acids, the approximate composition of pig and similar fats. The process has the great virtue of dispensing with the laborious ester fractionation and can therefore be used as a routine method and requires relatively small samples.

FATS OF AQUATIC ORIGIN

The alcohols present in the oil from the bottle-nosed whale have been examined by Tsujimoto (114), who found that they were the same as those of the sperm whale. The chief oxidation products obtained from the acetylated alcohols were *n*-nonanoic and acetyl-hydroxynonanoic acids. The main constituent of the alcohols must therefore have been $\Delta^{9:10}$ oleyl alcohol corresponding to oleic acid. The same author (115) has also found the alcohols of karasumi oil to be mainly $\Delta^{9:10}$ hexadecenol with smaller amounts of the C_{18} homologue. A general analysis of a whale blubber oil is reported by Glimm & Giese. Acids of the C_{2n} series from C_{12} to C_{22} were found to be present, the higher unsaturated members having four and five double bonds.

The characteristics of oils obtained from many different parts of the bodies of the humpback and blue whales have been recorded by

Ueno & Iwai. The entrail and lip skin fat are amongst the most saturated, while that of the fatty layers and flesh are most unsaturated. For the humpback whale the iodine value ranges from 122.6 to 148.9 and for the other from 94.9 to 129.5. The refractive index of the different fats changes with the iodine value and falls from 1.4690 to 1.4650 for the humpback whale and from 1.4660 to 1.4619 in the other. In this connection Harrison *et al.*, analysing salmon oil, found a positive correlation of 0.9749 between the iodine value and refractive index for the oil from this particular fish, and were able to estimate the iodine value from the formula:

$$\text{Iodine value} = 6,929 n_{D}^{25} - 10,079.2$$

with a standard error of ± 5.23 . The authors come to the conclusion that chemical and physical variations in salmon oils are larger than in fish oils of other types. Thus the iodine value of the oil from the body trimmings varied between 100 and 170, and was 220 for the oil from the salmon eggs. A study of these variations suggested that they are due to changes in the unsaturated acids rather than in the saturated constituents.

The highly unsaturated acid ($C_{24}H_{38}O_2$) from the oil of the tunny fish has been the subject of research by Ueno & Takase. Fractions (b.p. 224 — 6°/3 mm.) were isolated, which, on oxidation with ozone, yielded propionaldehyde, acetaldehyde, and succinic acid. The authors conclude that the acid contained the group $CH_3 \cdot CH_2 \cdot CH:$ with either $:CH \cdot CH_2 \cdot CH:$ or $:CH \cdot CH_2 \cdot COOH$ and either $:CH \cdot CH_2 \cdot CH_2 \cdot CH:$ or $:CH \cdot CH_2 \cdot CH_2 \cdot COOH$. A similar type of acid with 22 carbon atoms from cod-liver oil has been studied by Farmer & van den Heuvel. The highly unsaturated acid fraction obtained from about 6 kg. of the oil was converted to methyl esters and subjected to evaporative distillation at a pressure of about 10^{-4} mm. and a maximum temperature of 110°. The total time of contact with the heated surface was only about sixty seconds, so that the possibility of polymerisation or decomposition of the highly unsaturated esters was minimised. A fraction was obtained that weighed 225 gm. and contained only the ester of a C_{22} acid with six double bonds. By hydrogenation this ester was converted to methyl behenate which on saponification gave behenic acid of more than 99 per cent purity as determined by its x-ray analysis. Oxidation of the unsaturated ester gave rise to carbon dioxide, acetaldehyde, succinic

acid, methyl hydrogen succinate, and acetic acid. It was therefore concluded that the original acid had one of five formulae, all composed of one $\text{:CH}\cdot(\text{CH}_2)_2\cdot$ and four $\text{:CH}\cdot\text{CH}_2\cdot\text{CH:}$ groups arranged between the two terminal groups $\text{CH}_3\cdot\text{CH:}$ and $\text{:CH}(\text{CH}_2)_2\text{COOH}$.

Lovern has recently analysed the fatty acids contained in the body fats of four species of sea bird, the herring and skua gulls, the gannet, and the fulmar petrel. All these birds were obtained from a locality in which their diet would be most likely to consist entirely of fish or other aquatic life. The saturated fatty acids consisted of decanoic, up to 0.3 per cent; myristic, 2.0 to 3.3; palmitic, 13.9 to 18.5; stearic, 3.2 to 6.2; and arachidic, up to 0.2 per cent; the unsaturated acids were 0.4 to 1.0 per cent of C_{14} , 3.9 to 5.2 of C_{16} , 26.9 to 32.6 of C_{18} , 19.7 to 26.8 of C_{20} , and 16.5 to 22.1 of C_{22} with a possible trace of C_{24} acids, the degree of unsaturation of the liquid acids being high but not so high as in fish. The fats of the birds were therefore similar to the fats on which they would feed except that they had become slightly more saturated. Hilditch & Lovern had previously published a most interesting article showing the broad relationship generally existing between species on the one hand and fat composition on the other. Lovern notes that the present instance is an exception to this rule and that for the fat of these four sea birds this phytogenetic relationship does not hold. He suggests that this exception is either due to the fact that the birds have no specific requirements for their depot fat, with the result that its form can vary within very wide limits and therefore naturally assumes the characteristics of the diet, or that as the birds evolved the depot fat was gradually modified to suit that of the normal diet. The body fat of the penguin has been studied by Ueno & Aoki. It was somewhat similar to that of the sea birds just mentioned in containing 4 per cent of myristic acid, 14 of palmitic, 5 of stearic, and some highly unsaturated acids akin to those of fish.

The fats of sea algae are being investigated by Takahashi *et al.*, who have recently worked out the composition of the fatty acids derived from *Cystophyllum hakodatense* Yendo. They contain 22.9 per cent of solid acids made up of myristic and palmitic in the ratio of one to four. The unsaturated acids contain 47 per cent of oleic acid, 28 per cent of C_{16} compounds with one and two double bonds, 14 per cent of C_{18} acids with two, three, and four double bonds, and 5 per cent of C_{22} acids with two and three double bonds.

THE FATTY CONSTITUENTS OF INSECTS

The fatty constituents of Japanese wild bees and their combs have been investigated by Ueno & Komori. The comb oil fatty acids were chiefly palmitic, oleic, and linoleic while in the unsaponifiable matter, oleyl alcohol, and hydrocarbons were identified. Attention is drawn to the fact that the oils of wild bee combs consist of C_{16} and C_{18} acids while the common beeswax contains higher saturated acids. Beeswax from Formosan bees, collecting honey mainly from citrous trees, has been investigated by Hata (43). The fatty acids were mainly palmitic with small amounts of oleic and higher fatty acids. The unsaponifiable matter containing various higher alcohols and hydrocarbons amounted to 80 per cent of the wax. Another insect fat, that of adult South American locusts, has been studied by Trevithick & Lewis.

THE LIPOIDS OF MICROORGANISMS

Anderson and his colleagues have continued their work on the lipoids of bacteria. One paper published recently (3) deals with the α - and β -mycolic acids of avian tubercle bacilli. These substances are non-crystalline hydroxy compounds of very high molecular weight which are not readily purified. Nothing is yet known of their structure but on distillation at 210° to 225° (1 mm. pressure) the α -acid decomposes and yields 25 per cent of a pentacosanoic acid ($C_{25}H_{50}O_2$) with a side chain in its molecule, the residue consisting of a non-volatile substance with a molecular weight of about 1000. The β variety heated to 280° to 295° (1 mm. pressure) gives 21 per cent of *n*-tetracosanoic acid.

Akasi (1) has examined the ether extract made from typhoid bacillus of mice. He concluded that the fatty acids were made up of 51 per cent of solid acids consisting mainly of palmitic with some myristic and lauric, and 46 per cent of liquid acids, chiefly oleic with some palmitoleic. Linoleic, linolenic, and stearic acids appeared to be absent.

Chargaff & Levine have studied the lipoids of *Phytomonas tumefaciens*, the bacteria responsible for the growth of crown-galls in plants. In the acetone-soluble material they detected the presence of some 13 per cent of a liquid saturated acid having a formula corresponding with $C_{21}H_{42}O_2$. Palmitic, stearic, and oleic acid were also present. The existence of a liquid saturated acid in the phosphatide fraction of the same bacteria even when grown on different media is

also recorded by Geiger & Anderson. On a glycerol-containing medium the bacteria yielded 2 per cent of lipoids, of which 44 per cent was phosphatide, whereas on a sucrose-containing medium the lipid content was 6 per cent, and of this 64 per cent was phosphatide. For both types of medium the phosphatides were made up of almost equal parts of lecithin and cephalin, but the fatty acids obtained from the two types varied. With the sucrose media, the chief acids present were liquid unsaturated compounds accompanied by smaller amounts of palmitic, stearic, and liquid saturated acids. With the glycerol medium on the other hand, liquid saturated acids were the main components along with smaller quantities of the others. The nature of these liquid saturated acids is not known but it was clear that they differed considerably when grown on different media.

The lipoids of the pathogenic fungus, *Monilia albicans*, have been discussed by Peck & Hauser. From the dried cells they extracted 5.3 per cent of fat-soluble material and found it to consist of 3 per cent of phosphatides and 97 per cent of an acetone-soluble fraction. The latter contained 79.5 per cent of fatty acids and 13.6 per cent of unsaponifiable matter of which a large part was ergosterol. The approximate composition of the acid fraction as calculated from the equivalent weights and iodine values of the solid and liquid acids was found to be palmitic acid, 15.5 per cent; stearic, 5.1; oleic, 48.6; and linoleic, 10.3 per cent. The results are compared by the authors with those which they had previously obtained for *Blastomyces dermatiditis*.

WAX COMPONENTS

Apart from the beeswax already mentioned, one of the few wax-like substances which have been studied in the past year is that of the sugar cane, which has been investigated by Vidyarthi & Narasingarao. It yielded 43.7 per cent of acids and 53.6 per cent of unsaponifiable matter. The fatty acids consisted mainly of palmitic, 27.7 per cent; stearic, 22.4; oleic, 41.5; and arachidic, 3.3 per cent. By separating some of the constituents of the unsaponifiable matter with phthalic anhydride the authors concluded that it contained some 80 per cent of alcohols such as *n*-triacontanol and 5 per cent of hydrocarbons such as *n*-pentatriacontane. Similar long chain aliphatic alcohols present in the African palm have been discussed by Ungnade.

The constitution of lanopalmic acid from merino wool wax (m.p. 86–87°, $[\alpha]_D -1.5^\circ$) has been the subject of researches by Kuwata.

On mild oxidation, the acid yielded $n\text{-C}_{14}\text{H}_{28}\text{CHO}$, while with chromic acid the methyl ester gave an α -ketonic ester which was converted by further oxidation with hydrogen peroxide to $n\text{-C}_{14}\text{H}_{28}\text{COOH}$. The lanopalmic acid was therefore concluded to be *l*- α -hydroxypalmitic acid.

LIPOID DISTRIBUTION

Several papers have been published in the past year dealing with the types of lipoid found in different tissues of plants and animals but emphasising more their general distribution than the actual chemical nature of their components. Thus for ivy, Ulrich has shown that the green pericarp contains almost all the phosphatide of the fruit, but a very much smaller proportion of glycerides than the seeds. Another communication by Sullivan & Howe deals with the lipoids of wheat flour as compared with those of the germ. They state that the amount of volatile soluble fatty acid and the content of unsaponifiable matter are greater in the flour than in the germ.

On the animal side Koppenhoefer (73) who has been studying the lipoids of fresh and cured animal skin has now made a detailed analysis of the lipoids from the wool, the epidermal division, corium, and subcutaneous tissue of sheep. He finds a greater concentration of lipoids in sheep skin than in those of the goat or steer, and of the total lipoids 75 per cent are made up of waxes. The lipoids of the epidermal layer have been separated into wax, phosphatides and acetone-soluble fractions, but the chief components are wax and cholesterol. The lipoids of the corium and subcutaneous tissue on the other hand are mainly triglycerides. A description has also been given of the effect of the various curing processes on the lipoids of sheep skin where perhaps the chief change observed is the loss of 50 per cent of the epidermal lipoids (74). Again for fresh seal skin, Partridge has shown that the lipoids of the corium are mainly triglycerides but that the fatty substances of the epidermal layer contain large proportions of highly unsaturated hydrocarbons and cholesterol. He has concluded that muscular exertion by the animals before killing is accompanied in the epidermis by the loss of triglycerides, an increase in phosphatides, and an increase in the degree of unsaturation of the lipoids.

The acetone-soluble and acetone-insoluble fractions of the lipoids of cell nuclei have been studied by Stoneburg. In the cell-nuclei of heart muscle, as in blood, the phosphatide fraction appears to contain

fatty acids which are much more saturated than those of the acetone-soluble portion.

In the analysis of blood lipoids the separation of cholesteryl esters has never been satisfactorily achieved. The use of castor bean lipase for effecting such a separation has now been studied by Kelsey. Under suitable experimental conditions he was able to obtain complete hydrolysis of tripalmitin in mixtures containing 40 mg. of the triglycerides, 40 mg. of cholesteryl ester and 20 mg. of free cholesterol. No cholesteryl ester was either hydrolysed or synthesised. Kelsey has also prepared an enzyme with the same specificity from the pancreas by using, as an extracting agent, dilute ammonia which destroys the activity of the cholesterol esterase but leaves that of the lipase relatively unimpaired. He has applied these two lipases to the hydrolysis of the acetone-soluble lipid fraction in dog plasma and ox serum but finds that only 50 to 60 per cent of the triglycerides calculated to be present are hydrolysed. He therefore suggests that either the lipase does not act with blood triglycerides as it does with tripalmitin or that esters of unknown nature are present in the so-called neutral fat of blood. He states that while the average molecular weight of the hydrolysed glyceride fatty acids from ox serum suggested that they were mainly of the C_{18} type, those of the cholesteryl ester fraction appeared to have a mean molecular weight of only 227, indicating that they contained considerable quantities of lower fatty acids. This, in Kelsey's view, may connect them with milk fat secretion. It should be noted, however, that Smith (102), investigating the plasma lipoids of the lactating cow, found mean molecular weights of about 300 for the fatty acids from the acetone-soluble fraction, which would suggest the presence of higher, rather than lower, acids in the cholesteryl esters.

THE PHOSPHATIDES

A series of three papers has been published by Kabashima on the synthesis of the phosphatides. In the work described in the first of these, glycerylphosphoric acid was purified in the form of its barium salt and converted by means of palmityl chloride to $\alpha\alpha'$ -dipalmitylglyceryl- β -phosphoric acid. The silver salt of this compound then reacted with β -bromoethylamine picrate in chloroform-acetone solution to give $\alpha\alpha'$ -dipalmitylglyceryl- β -kephalin and with bromocholine picrate to give the corresponding β -lecithin. The second paper deals with the synthesis along similar lines of α -kephalin distearate which

is stated by the author to be similar to the type of natural product obtainable from human brain, while the third paper describes the synthesis of natural lyso-lecithin. The haemolytic power of the synthetic lyso compound was rather less than that of the product derived from egg yolk. Lysophosphatides have also been discussed by Chargaff & Cohen.

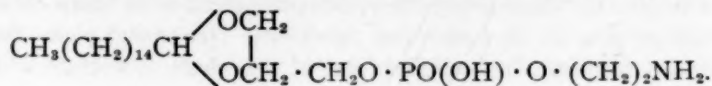
Synthetic lecithin-proteins have been made by Hofer by adding a solution of lecithin in alcohol to a solution of serum albumin. On adding ammonium sulphate or acetic acid a serum albumin-lecithin complex was obtained which was readily soluble and could not be broken down by ether extraction. It has also been shown that although lecithin passes quantitatively into the ether layer when alcohol and ether are added to a lecithin solution at pH 2.5, only traces dissolve in the ether at pH 3.0 when 1 per cent of ovalbumin is present. The liberation of phosphatides from certain protein complexes of serum by means of soaps has been studied by Macheboeuf & Tayeau. They find that the phosphatides can be removed from the globulin fraction without denaturation by using potassium dibromostearate.

The phosphatides of lupins have been studied recently by Die-mair & Weiss. They found them to consist of 26 per cent kephalin and 74 per cent lecithin. There appears to be some 50 per cent more α - than β -glycerophosphoric acid in these lipoids.

Methods of phosphatide analysis have been discussed by Williams *et al.* and Thannhauser and associates (110). The same problem has also been discussed by Teunissen & den Ouden, and an apparatus for the estimation of as little as 5 gm. or less of methyl esters prepared from phosphatide fatty acids is described by Die-mair & Schmidt.

With regard to the cerebroside, papers have recently appeared by Klenk & Clarenz on phrenosinic acid, and by Müller on phrenosinic and nervonic acids.

During the past year three papers have been published by Feulgen *et al.* on a new group of phosphatides which have been named "plasmalogens." These substances are acetal phosphatides and on hydrolysis they yield colamine esters of α - and β -glycerophosphoric esters along with 55 per cent of a mixture of aldehydes to which the name "plasmal" has been given. α -Palmitalplasmalogen is probably



A method is described for determining the amount of plasmal in phosphatides, and values are given for those of muscle, 10 to 12 per cent; brain, 8 to 10; liver, 1; egg, less than 0.1 per cent. Brain and muscle plasmal consist mainly of C_{16} and C_{18} saturated aldehydes, but unsaturated compounds of a similar nature may also be present.

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THE CHEMISTRY OF THE LIPINS

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Since the appearance of the last review on this subject an excellent survey has been given by Page (1) on various problems related to phospholipid chemistry. The present review covers the period up to and including August 1939. A special consideration has been given to progress in the field of phospholipid analysis and metabolism.

METHODS OF QUANTITATIVE DETERMINATION

Estimation of total phosphatide.—The general opinion held that a large proportion of the alcohol-ether soluble phosphorus is not lipid in character has been questioned by several investigators. Thus, Man (2) succeeded in recovering 95 to 100 per cent of the alcohol-ether soluble phosphorus in the petroleum ether solution of the lipid residue, when the evaporation of the alcohol-ether extract was carried out *in vacuo* in the presence of an inert gas and at temperatures not exceeding 37°. Essentially similar results were obtained by Ellis & Maynard (3) and by Williams *et al.* (4). The influence of the temperature on the resolution of the phosphatides in petroleum ether has previously been demonstrated by Kirk, Page & Van Slyke (5) and their conclusions confirmed by Brun (6). From her results Man considers it justified to omit re-resolution in petroleum ether and to calculate the phosphatide content of blood and organs from determination of the alcohol-ether soluble phosphorus. According to Man & Gildea (7) higher yields of phosphatide were obtained from whole blood (up to 0.4 mg. per cent of lipid phosphorus) and to a smaller extent from serum, when the alcohol-ether mixture was refluxed for one hour than when the Bloor extraction method was used. Boyd (8) found a significant decrease in the phospholipid content of stored samples of alcohol and alcohol-ether extracts when the phosphatide determinations were performed by the oxidative procedure. The decrease began to occur after one month's storage, but a significant reduction was not found until after three months, at which time the average phospholipid content of the samples had fallen by 30 per cent. The method of storage—whether in the light or dark, at room temperature or in the refrigerator, in alcohol or in alcohol-ether—did not materially in-

fluence the result. As no significant change was observed in the amount of total fatty acids in the extracts it was concluded that the loss of phosphatide was due to hydrolysis and not to oxidation. As suggested by Boyd the low blood phosphatide values reported by Corcoran & Rabinowitch (9) from analyses on extracts stored for a period of several months may be explained by such hydrolysis.

Estimation of the individual phosphatides.—Procedures for determination of the individual phosphatides in a lipid mixture have been reported by several authors. Although the methods suggested still await further trial for full appreciation of their value and applicability it already appears unquestionable that considerable progress has been obtained in this field.

In uncontaminated phosphatide samples cephalin can be titrated in benzene with absolute alcoholic sodium hydroxide and phenolphthalein (10), the result paralleling those obtained by Van Slyke's gasometric method. Attempts to determine cephalin directly in petroleum ether lipid extracts by amino nitrogen analysis on the emulsified residues have proved unsuccessful because of the inclusion of large amounts of nonphosphatide amino nitrogen in the extracts (5). The nature of the nitrogenous contaminants has been subjected to a detailed study (11) and urea identified as the chief constituent (12, 13). In a series of analyses of normal and pathological serum samples urea was found to represent 37 to 82 per cent of the extra nitrogen; the plasma lipids apparently lend to petroleum ether an ability to dissolve urea which petroleum ether by itself does not have. Smaller amounts of amino acid nitrogen were also demonstrated in the petroleum ether extracts by the gasometric carboxyl method of Van Slyke & Dillon [see Folch & Van Slyke (12)]. A great part of the nonphosphatide nitrogen can be removed by shaking with acidified water (11) or by precipitation with colloidal iron (14). Schmitz & Koch (15) claim that the contaminating amino nitrogen can be removed quantitatively from the petroleum ether extracts by shaking with 0.01 *N* sulfuric acid and 20 per cent magnesium sulfate; Kirk (16) was only able to remove up to 50 per cent of the amino nitrogen by this treatment. The amount of urea carbon entrained with the lipids in the petroleum ether extracts was not found sufficient to affect significantly the total lipid values determined by the gasometric method (12).

Lintzel & Fomin (17) and Lintzel & Monasterio (18) destroyed the choline of the phosphatides by boiling with strong alkali and estimated the liberated trimethylamine by formalin titration. This proce-

dures afforded no separation between lecithin and sphingomyelin, but made possible the indirect determination of cephalin by subtraction of the choline-containing phosphatide value from the amount of total phosphatide. For accurate analysis the procedure requires choline samples as large as 2 to 5 mg. Williams *et al.* (4) used the same indirect method of cephalin calculation, but substituted Beattie's colorimetric method (19) for determination of choline.

An approximate separation of lecithin, cephalin, and ether-insoluble phosphatide by microanalysis was attempted by Kirk (16, 20). The phosphatides were precipitated by acetone and magnesium chloride according to Bloor and the precipitate treated with moist ether for re-solution of lecithin and cephalin. Lecithin was estimated in the moist ether extract by saponification with barium hydroxide and subsequent choline analysis according to Roman (21). Cephalin was calculated as the remaining ether-soluble phosphatide, estimated by gasometric carbon determination or by phosphorus analysis of the residue of an aliquot of the ether extract. The amount of ether-insoluble phosphatide was determined by phosphorus analysis of the fraction which did not dissolve in moist ether. In analyses of lecithin samples of 0.5 to 5.0 mg. the error rarely exceeded 5 per cent. Mixtures of standard lecithin and cephalin samples were likewise accurately determined. The separation of sphingomyelin from the glycerophosphatides appeared to be nearly complete under the conditions of the method, but it was doubtful whether the ether-insoluble phosphatide fraction represented sphingomyelin only. Blix determined the glycerol (22), phosphorus, and choline content of the phosphatides after isolation and from these values was able to estimate the lecithin, cephalin, and sphingomyelin concentrations (23). In the procedure of Thannhauser & Setz (24) and Thannhauser, Benotti & Reinstein (25) sphingomyelin is precipitated as the reineckate and determined gravimetrically, lecithin is estimated by subtracting the sphingomyelin value from the total amount of choline-containing phosphatide, determined by the method of Beattie after hydrolysis with gaseous hydrogen chloride in absolute methanol, and cephalin is calculated as the remaining phosphatide. For determination of the sphingomyelin content of serum the method requires samples as large as 50 cc. According to Thannhauser, Benotti & Reinstein only that portion of the reineckate which is insoluble in cold acetone can be considered as sphingomyelin (and not as previously stated also the acetone-soluble reineckate). This correction makes the first sphingomyelin values reported by Thann-

hauser & Setz notably higher than the values published later (see Fig. 1). The direct analysis of sphingomyelin by an easily controlled chemical procedure, as introduced by Thannhauser & Setz, undoubtedly represents the most valuable contribution to the quantitative determination of the individual phosphatides.

Estimation of cerebrosides.—Kimmelstiel (26) determined cerebrosides by acid hydrolysis and subsequent titration of the liberated galactose. Similar procedures have previously been employed by Noll (27) and by Winterstein & Hirschberg (28). In the original method of Kimmelstiel the initial reduction value of the lipid mixture was subtracted from the value found after hydrolysis, this feature notably reducing the cerebroside figures obtained on analysis. Kimmelstiel later (29) introduced another change in the technique by precipitating the unhydrolyzed lipid samples with zinc hydroxide previous to the determination of the initial reduction value. As demonstrated by Kimmelstiel, and confirmed by Kirk (30), such precipitation causes a marked decrease in the initial reduction figures, the values found in analyses of alcoholic brain extracts being only about 15 per cent of the values obtained from untreated samples. The failure of Kimmelstiel to subject also the hydrolyzed lipid samples to zinc precipitation again resulted in cerebroside values which were too high. A modified procedure suggested by Kirk (30) includes zinc precipitation of both unhydrolyzed and hydrolyzed samples previous to determination of the reduction values. By this change of technique cerebroside values, only 30 to 70 per cent of those found by Kimmelstiel's (29) procedure, were obtained in analysis of brain material. The modified method which also includes a simpler procedure for acid hydrolysis permits determination of pure cerebroside samples in amounts varying from 0.3 to 1.3 mg. with an average error of 4 per cent, and likewise permits the quantitative recovery of cerebrosides added to alcoholic brain extracts. It appears doubtful whether the values found in analysis of blood and tissue extracts represent the true cerebroside figures, as erroneous findings may result from a change in the reduction values of interfering substances during the acid hydrolysis. Fawaz, Lieb & Zacherl (31) precipitated the brain material with trichloroacetic acid previous to extraction with hot alcohol. This treatment was not found to interfere with the quantitative cerebroside extraction, but caused removal of other reducing substances. Kirk later (32) adapted the colorimetric orcin reaction, developed by Tilman & Philippi (33), in the modification of Sørensen & Haugaard

(34), to direct determination of the carbohydrate content of the lipid mixture. This technique also yielded quantitative results in analysis of pure cerebroside samples but, in analyses of petroleum ether lipid extracts of plasma and erythrocytes, usually gave values considerably higher than the titrimetric procedure. This discrepancy may be due to the presence of carbohydrates other than galactose in combination with the lipids, as previously suggested by Bing (35, 36).

Thompson & Wright (37) have made preliminary studies on a colorimetric method for cerebroside determination. On addition of sulfuric acid and chloroform to cerebroside a purple color develops, which passes into the chloroform layer. This reaction, which appears to be similar in nature to the Molisch reaction, has been described before by Thudicum. The intensity of the produced color was found to bear a linear relationship to the amount of cerebroside present. Other substances present in the alcoholic tissue extract did not appear to interfere with the reaction. If further developed the method might prove a valuable aid in the quantitative cerebroside determination.

THE PHOSPHOLIPID CONTENT OF BLOOD AND ORGANS UNDER NORMAL AND PATHOLOGICAL CONDITIONS

Blood and lymph.—The phospholipid content of serum of normal adults was studied by Man & Gildea (38) over periods up to four years. A maximum variation of 23 per cent was found in the concentration of serum phospholipids of the individual subject. The changes were unrelated to the menstrual cycle and to the season of the year. In an investigation of twenty normal Danish adults Kirk (39, 40) found total phosphatide values of plasma only half of those found by a similar technique used in a study of healthy Americans (41). Estimations of the total phosphatide concentration of the erythrocytes in a larger group of normal children (42) and adults (39, 40) have been reported for the first time. The average concentration in the (American) children was 246 mg. per cent (21 analyses), and in the (Danish) adults 196 mg. per cent (20 analyses). Several attempts have been made, for the first time on a large scale, to determine the concentration of the individual phosphatides in human plasma and red blood cells. A summary of the findings of recent years is given in Fig. 1. It may be seen from the figure that cephalin constitutes an important fraction of the phosphatides in both plasma and cells and that notable amounts of sphingomyelin are likewise present. According to Thannhauser, Benotti & Reinstein (25) the concentration of lecithin in

plasma is also significant. Other investigators were, however, unable to demonstrate larger amounts of this phospholipid in plasma (23, 40) and erythrocytes (43, 44). The average concentrations found in twenty normal individuals for lecithin, cephalin, and ether-insoluble

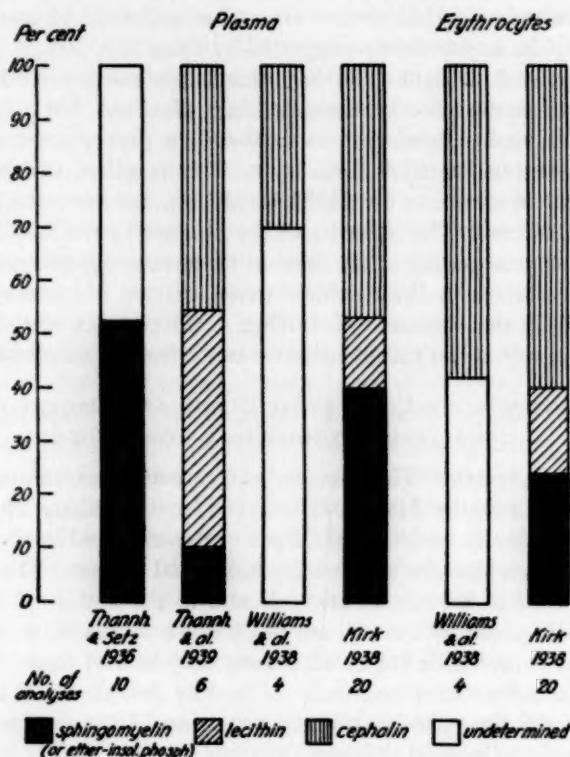


FIG. 1.—The percentage concentration of the individual phospholipids in human plasma (serum) and red blood cells

phosphatide were 19, 68, and 58 mg. per cent respectively in the plasma, and 32, 117, and 47 mg. per cent in the cells. In eight of the twenty analyses the lecithin concentration in the cells was less than 10 mg. per cent (40). The considerable variation found between different individuals may partly be attributable to some inaccuracy in the available analytical technique.

Direct evidence of the presence of sphingomyelin in cow's plasma

was obtained by Ellis & Maynard (3) by examination, between crossed Nicol prisms in the polarizing microscope, of a fraction insoluble in ether. The sphingomyelin isolated from bovine blood cell stroma was found to consist chiefly of lignoceric sphingomyelin (45).

Boyd (46) has continued his studies on the phospholipid content of leucocytes and reports an average phosphatide concentration of rabbit white blood cells of 864 mg. per cent (244 to 1310 mg. per cent). According to Erickson and co-workers (47, 48) dried human blood platelets contain about 12 per cent of phosphatides (of a total lipid content of 16 per cent), 68 per cent of which is cephalin. The platelets from normal children are lower in phospholipids. The presence of both lecithin and cephalin in horse platelets has previously been demonstrated (49). In the thoracic lymph from a patient on a rigid low-fat diet Reiser (50) found a phospholipid content of 68 to 84 mg. per cent, a figure which is in good agreement with those of previous observers.

In hemolytic and hypochromic anemias of childhood the phospholipids of plasma showed no definite variations. In the cells low phosphatide values were found in hemolytic jaundice, but increased values in hypochromic nutritional anemias both before and after treatment (51). The same authors failed to observe any significant changes in the phospholipids of the cells in erythroblastic anemia, whereas slight increases were reported in this disease by Whipple & Bradford (52). In pernicious anemia of adults all investigators (39, 53, 54, 55, 56, 57) are agreed that the phospholipid values of serum are frequently greatly reduced in untreated cases, but tend to increase toward normal during spontaneous remission or as the result of effective treatment. Changes in the ether-insoluble phosphatide fraction were chiefly responsible for the plasma phospholipid variations (39, 57). In nine out of ten patients subjected to liver treatment a marked increase in this phosphatide fraction was found to occur during the treatment. The increase was noticeable after two to four weeks' treatment with liver extract, thus occurring somewhat later than the reticulocyte response. After five to six months the values of ether-insoluble phosphatide again approached the normal figures. No consistent changes were noted in the cephalin or lecithin concentrations, nor was any difference observed between the uncomplicated anemia cases and the patients presenting spinal involvement (57). In two cases of untreated pernicious anemia the intramuscular injection of egg phosphatide caused no reticulocyte response or increase in red blood cells (32).

In eighteen cases of disseminated sclerosis Calvary (58) found normal values of total plasma phosphatide and lecithin. Determinations of cephalin and ether-insoluble phosphatide were not reported.

Analyses of the phospholipid content of blood platelets from hemophilic patients indicate that the total phospholipid values are somewhat lower than normal and the proportion of neutral fat higher. The percentage of phospholipid present as cephalin appears to be normal (47, 48).

Fever in twenty-four rabbits infected with *Streptococcus viridans* caused a several hundred per cent increase of the phospholipid content of plasma, which lasted for two weeks. The changes in the erythrocytes were insignificant. The infection also produced a marked secondary anemia which may have been a contributory cause of the lipemia (59). In acute infections of infants and older children the phospholipid content of serum was found decreased (60).

Organs.—Analyses of the lecithin, cephalin, and sphingomyelin content of various human organs were reported by Thannhauser *et al.* (61) (see Table I). As is evident from the table the cephalin content of the brain is considerably higher than the lecithin concentration,

TABLE I
THE LECITHIN, CEPHALIN, AND SPHINGOMYELIN CONTENT OF
NORMAL HUMAN ORGANS*

Organ	Lecithin†	Cephalin†	Sphingomyelin†
Brain	4.81	20.42	5.66
Lung	3.85	2.00	1.45
Spleen	3.54	4.16	0.86
Kidney	5.10	3.26	0.72
Liver	4.81	4.62	0.38
Heart	4.47	2.06	0.34

* From Thannhauser *et al.* (61).

† The values represent mg. per 100 mg. of dried organ.

whereas in liver and spleen the two phosphatides were found to occur in almost equal amounts. In kidney, heart, and lung, lecithin predominates in a proportion of about two to one. From the gastric mucosa of pigs both cephalin and lecithin have been isolated, whereas the presence of sphingomyelin could not be demonstrated (62). An extensive study of the lipid distribution in fresh steer skin was accomplished by Koppenhoefer (63). The maximum phospholipid concentration was found in the rapidly multiplying cells of the basal layers

of the epidermis, whereas appreciably lower values were demonstrated in the corium and the horn division. In the lipids isolated from the hairs phospholipids were lacking completely. A similar decrease in phospholipid has been observed by Kooyman (64) during keratinization in human skin. It therefore appears evident that the phospholipid molecule is destroyed during keratinization. In several samples of steer skin a sphingomyelin-like body was present in the region between the epidermis and the corium.

Randall (65) and Yasuda (66) found a higher phospholipid content in the white areas of the brain than in the grey. The iodine number of the phospholipid fatty acids was higher in the grey than in the white tissues (65). In the peripheral nerves from arteriosclerotic and diabetic subjects (67, 68, 69) the phospholipid concentration was markedly lower (1.2 to 2.7 per cent of moist weight) than in the normal nerves (3.9 to 4.6 per cent), the chemical changes being more pronounced in the distal than in the proximal parts in accordance with the severity of the histological changes (70). After section of rabbits' nerves a similar decrease in the lipid phosphorus content occurs (71).

Tötterman & Kirk (72) studied the lipid composition of six fresh specimens of *Botriocephalus latus* obtained from patients suffering from hyperchromic anemia. In agreement with the earlier observations of Faust & Tallquist (73) considerable amounts of phospholipid were found. The average lipid concentration in the fresh worms was 1.6 per cent and the phospholipid content 0.57 per cent. The cephalin fraction constituted the major part of the phosphatides (0.36 per cent), but appreciable amounts of ether-insoluble phosphatide (0.08 per cent) and lecithin (0.13 per cent) were also demonstrated.

THE CEREBROSIDE CONTENT OF BLOOD AND ORGANS UNDER NORMAL AND PATHOLOGICAL CONDITIONS

Kirk (40) determined the cerebroside concentration of human plasma and blood cells using the modification of Kimmelstiel's procedure described above. In plasma the values were found to vary greatly (0 to 167 mg. per cent). In the cells the variations, though considerable, were somewhat smaller, the average concentration being about 50 mg. per cent. In seven out of fourteen untreated cases of pernicious anemia the cerebroside were lacking in the cells, but appeared after liver treatment (57). In interpretation of this observation it should be recalled that the procedure used is probably not quite specific and

that the concentration in normal erythrocytes varies greatly. Nevertheless it would appear that the cell constituent in pernicious anemia may frequently be abnormal, a fact which is of interest in view of the demonstration by Bang & Ørskov (74) of an abnormal permeability of the erythrocytes in this disease.

Thannhauser, Benotti & Reinstein (25) were unable to isolate cerebrosides from large volumes of bovine plasma. Small amounts of cerebrosides were found in the gastric mucosa of pigs (62). The presence of cerebrosides was also demonstrated in the brains of various fish (30) and in the *Botriocephalus* specimens analyzed by Tötterman & Kirk (72). Among the invertebrates, only *Octopus vulgaris* failed to show cerebrosides in the nervous tissue. In the vertebrates the cerebroside concentration generally was found to increase with phylogenetic evolution (75). Using the original Kimmels procedure Randall (69) observed a marked reduction in the cerebroside content of peripheral nerves of arteriosclerotic and diabetic subjects (from 1.4 to 0.6 per cent of moist weight); the decrease occurred simultaneously with the phospholipid depletion. The reduction appeared to be the result of autolytic processes in the degenerating nerves.

PHOSPHOLIPID AND CEREBROSIDE METABOLISM

The introduction of labelled atoms or groups (radioactive phosphorus, deuterium, iodized fatty acids, elaidic acid) into the phospholipid molecules has greatly increased the knowledge of the physiology and function of these substances. The use of radioactive phosphorus has proved especially advantageous in the study of phospholipid metabolism. The applicability of this indicator is partly dependent on its convenient period of decay (half-life time about two weeks). Also the identification of the phospholipids by radioactive phosphorus possesses advantages over methods in which a foreign fatty acid is employed, as the phosphatide molecules do not change their character to any noticeable extent by the introduction of the phosphorus isotope. As the phosphorus present in the phosphatide molecule can not be replaced by a simple exchange process (76) the presence of labelled phospholipids is a proof that a synthesis of phosphatide has taken place after the administration of the radioactive phosphate. The use of this indicator, therefore, makes it possible to measure the rate of formation of new molecules.

A quantitative comparison of the phosphatide turnover in differ-

ent organs by using radioactive phosphorus as an indicator has been made by several investigators [Artom *et al.* (77, 78, 79, 81), Hevesy *et al.* (76, 82, 83, 85, 87), Chaikoff *et al.* (80, 84), Dols *et al.* (86)]. In interpretation of the experimental results it should be considered that all the tissues in which labelled phosphatide appears may not be capable of such synthesis, as the phospholipids may be synthesized in another organ and transported elsewhere after formation (80).

Gastrointestinal tract.—The existence of a rapid turnover of phospholipids in the intestinal wall now appears to be definitely established. Thus it was found by Artom & Peretti (88) that the fatty acids of iodized fats when fed by mouth promptly enter into the phospholipids of the mucosa. After administration of elaidin to rats, between one-third and one-half of the natural fatty acids normally present in the mucosa phospholipids is replaced by elaidic acid (89). Later, Artom and co-workers, by using radioactive phosphorus, were able to demonstrate that the turnover of phospholipids involves a rapid change not only of the fatty acids of the molecules but of phosphoric acid as well (77). The formation of lecithin was found to proceed at a greater speed than that of cephalin (90, 91). In comparison with the small intestine, the stomach and the large intestine showed little activity (92, 93). Phospholipid formation is not confined to animals ingesting fat; formation during fasting amounts to about 30 to 40 per cent of that found during fat administration. It has not yet been settled whether this rapid turnover is an indication that the phospholipids are intermediary products in the resynthesis of absorbed fatty acids and glycerol, or whether it indicates that the phosphatides are synthesized in the intestinal mucosa and then distributed to the rest of the body through the blood stream.

Liver.—According to many observers (77, 79, 80, 82, 83, 84, 86) the liver is the site of the most active phosphatide exchange in the body. As the phosphatide turnover also takes place after removal of the gastrointestinal tract and the kidneys (92) it appears certain that this organ is capable of phospholipid synthesis. The importance of the liver for formation of the phosphatides is especially apparent from experiments on laying hens, which daily produce about 60 mg. of phospholipid phosphorus in the yolks; six hours after the administration of radioactive phosphorus to such animals 50 per cent of the labelled phosphorus was found in the liver (84). Also after feeding deuterium-containing fat to rats considerable amounts of deuterium were present in the liver lipids after this period of time (94). The

formation of phosphatides in perfusion experiments with isolated cats' livers was reported by Hahn & Hevesy (95). It was found that about 2 per cent of the phosphatides of the liver were renewed in the course of 2.5 hours when ordinary blood was used for perfusion. If lipemic blood was employed a greater synthesis was noted. This observation may explain, at least to some extent, the rise in the phospholipid concentration of the blood that occurs during alimentary lipemia, the increased phospholipid concentration in the liver causing an increased discharge of phosphatides into the circulation. In accordance with this concept, Hevesy & Lundsgaard (96) were able to conclude that the greater part of the phosphatides responsible for the phospholipid increase in plasma during alimentary lipemia are not synthesized in the intestine, as after ingestion of radioactive sodium phosphate together with oil the phosphatides extracted from the lipemic plasma were only slightly radioactive.

With the aid of radioactive phosphorus Perlman & Chaikoff (97) compared the rate of phospholipid turnover in the livers of choline-treated and untreated rats. A definite acceleration in the synthesis and transfer of the phospholipids was demonstrated, the difference between the labelled phosphatide content of the liver in the treated and untreated animals amounting to nearly 40 per cent. The change in phospholipid activity began approximately one hour after choline ingestion and lasted for about ten hours. In contrast to choline, cholesterol was found to decrease the phospholipid turnover in the liver appreciably, the depression being demonstrable as early as thirty hours after the feeding of cholesterol was initiated (98).

A comparison of the rates of formation of lecithin and cephalin in the liver was made by Chargaff (90, 91). It was found that the synthesis of lecithin was definitely greater than that of cephalin, as was also the uptake of elaidic acid by the lecithins [Sinclair (99)]. The difference in the fatty acid uptake was not sufficiently great to rule out the importance of the cephalins for phospholipid metabolism.

Blood.—Hahn & Hevesy (100) have studied phosphatide formation in the blood. A few cubic centimeters of dogs' blood were shaken with labelled sodium phosphate for about four hours. The phosphatides extracted after this period were only slightly radioactive, the amount of labelled phosphatide formed being only about 0.1 per cent of the total amount present. The appearance of notable quantities of labelled phospholipid in the blood after the administration of radioactive phosphorus therefore permits the conclusion that these phos-

phatides have been released from the organs in which they have been synthesized (101). On the other hand, labelled phosphatides introduced into the blood are taken up by the liver to a great extent (102). One day after the administration of radioactive phosphate the corpuscle phosphatides of human blood showed a radioactivity only one-fifth that of the plasma phospholipids. This finding excludes the possibility that the blood phosphatides are mainly formed in the erythrocytes (82, 83, 101).

A slight exchange of phosphatide molecules between plasma and corpuscles has been demonstrated. After shaking rabbit blood cells with rabbit plasma containing labelled phospholipids, 5 per cent of the erythrocyte phosphatides exchanged in the course of four hours (103). It is probable that only the phospholipids present in the outer layers of the cells exchange with those of the plasma. The greater part of the phosphatides present in the cells have presumably been incorporated in them during their formation.

The importance of plasma and blood cells for transporting phospholipids has continued to receive much attention. The significance of this transport mechanism is evidenced in the case of the laying hen which has to produce about 1500 mg. of phospholipid a day. As no appreciable phospholipid synthesis takes place in the ovary the phosphatides of the yolk must be extracted from the plasma, which contains a total of about 500 mg. of phosphatide. The equivalence of three times the total phosphatide content must therefore be given off from the plasma daily (83). In contrast to plasma the red blood cells were not found to participate appreciably in phospholipid transport; after administration of radioactive phosphate to hens the labelled phospholipid content of the erythrocytes was considerably less than that of the plasma (82, 83, 101).

The importance of the plasma phosphatides for the transport of fatty acids is apparent from the investigations of Sinclair (104). Following the administration of elaidin to cats considerable amounts of elaidic acid were found in the plasma phosphatides. After eight hours elaidic acid made up 15 per cent of the phospholipid fatty acids of plasma, whereas none was found in the phospholipids of the red blood cells. These findings support the conclusion that the corpuscles in contrast to the plasma do not take part in fatty acid transport to any great extent.

Brain.—In experiments on fully grown rats labelled phosphatide was demonstrated in the brain even one hour after the subcutaneous

injection of radioactive sodium phosphate (76). Similar results were obtained in adult mice and rabbits. Also by experiments *in vitro* a small but definite formation of phospholipid was found. These findings prove that a constant breakdown and building up of phosphatides takes place in the brain of fully grown animals, presumably under enzymatic action. The total phospholipid turnover in the brain is much smaller than that found in several other organs (76, 80, 105); it is approximately twice as great in young animals as in fully grown individuals (105). The radioactive lipid deposition in the brain reaches a maximum about 200 to 250 hours after the administration of labelled phosphorus; once the maximum amount of labelled phospholipid has become deposited its loss from the brain occurs at a very low rate, appreciable concentrations being found as late as four weeks after the administration (105). In rats provided with large amounts of elaidic acid throughout the entire period of prenatal and postnatal development the elaidic acid content of the lecithin and cephalin fatty acids of the brain was only about one-fourth that of the liver and muscles; this indicates a greater degree of selection in the building up of the brain phosphatides (106).

Muscle.—In accordance with earlier investigations in which a correlation was found between the activity of various groups of beef muscles and their phospholipid content Bloor (107) was able to demonstrate that experimentally increased activity of rats' muscles resulted in a rise in their phospholipid content. The effect of exercise was not reflected in the degree of unsaturation of the component fatty acids of the muscle phospholipids (108). No increase was observed in the concentration of phosphatides in the hypertrophied hearts of rats (109). In nutritional muscle dystrophy of rabbits the phospholipid content of the affected skeletal muscles was found higher than in the muscles of the normal animals (110).

Endocrine organs and mammary glands.—Kochakian *et al.* (111) found no correlation in castrated dogs between the plasma phosphatide level and the duration of the castration, nor was any change in the phospholipid concentration observed after testosterone injection. A three- to fourfold increase in the phosphatide content of hens' blood (up to 500 mg. per cent) at the time of production was ascertained by Heller *et al.* (112) and by Lorenz *et al.* (113). The high phospholipid concentration of plasma, which is of great importance for phospholipid transport in the animal during egg-laying, is maintained during the entire production period, but drops quickly as the molting

season approaches. The onset of ovarian activity, not yolk formation, was found to provide the stimulus for the rise in phospholipid content, as similar changes also occurred after prolonged administration of gonadotropic hormone (114). As mentioned previously the ovary does not appear capable of phospholipid synthesis to any great extent. The chief function of this gland seems to be the extraction of the phosphatides from the plasma and their combination with proteins for incorporation in the yolks (83). Although the phospholipid activity of the oviduct is four times greater per gm. of tissue in the laying than in the non-laying bird (93) no phospholipid formation could be noted in the eggs after they had left the ovary (83). In accordance with this no synthesis of labelled phosphatide was ascertained in experiments in which eggs were placed for a day in a solution containing radioactive sodium phosphate (83). Enzymes capable of phospholipid synthesis therefore appear to be lacking in the yolks. They are present in the embryo at an early stage.

Aten & Hevesy (115, 116) have made extensive investigations into the origin of the phosphatides in milk; they used radioactive phosphorus as an indicator. The phosphatides extracted from the mammary gland were found to possess a radioactivity about ten times greater than the milk phosphatides, which in turn showed a greater activity than the phospholipids of plasma. From these findings it may be concluded that the milk phosphatides are chiefly synthesized in the mammary gland, and are not derived, as are the yolk phosphatides, from the plasma phospholipids. Their formation in the mammary gland appears to be a slow process (116).

Embryo.—Hevesy, Levi & Rebbe (117) have studied the origin of the phosphorus compounds in the chick embryo. Radioactive phosphorus was introduced into fertilized hens' eggs. After the eggs had been incubated for various periods of time the radioactivity of the phosphatides of the embryo and yolk was compared; the former was found to be about one hundred times greater than the latter. From these results it follows that the phosphatide molecules in the embryo are not identical with those derived from the yolk, but are newly synthesized in the embryo. As inorganic phosphorus is present in the yolk in only small amounts, the inorganic phosphorus necessary for building up the skeleton and other organs of the chick is supplied from the phosphatides of the yolk, which gradually hydrolyze during the incubation period.

Tumors.—It has previously been found by Yasuda & Bloor (118)

that the phospholipid content of malignant tumors is two to three times that of benign tumors. In further work on this problem in Bloor's laboratory it was shown (119) that the phospholipid concentration in the outer actively growing portions of malignant rat tumors was much higher than in the inner slow growing or recessive parts. After elaidin feeding to rats inoculated with a carcinosarcoma, elaidic acid was found to enter the tumor to the extent of one-fifth of the phospholipids. The rate of entrance into and disappearance from the tumors was low compared with that for liver phosphatides (120). Similar conclusions were arrived at by the use of radioactive phosphorus as an indicator (87); the highest phospholipid activity observed in transplanted tumors of mice was approximately one-third of that found in the livers of normal animals (121).

Blood clotting.—The phosphatide fraction of horse blood platelets was shown to contain a potent activator for plasma clotting (49). The presence of unsaturated fatty acids in the cephalin molecule is important for the activating effect, lysocephalin lacking any influence on the blood clotting mechanism (122). The natural activator for blood clotting is now believed to be a complex between cephalin and a protein, probably specific in character (49, 123). The existence of a water-insoluble protamine salt of cephalin, consisting of about 80 per cent of cephalin and 20 per cent of salmine is reported by Chargaff (124). The compound was found inactive in blood clotting (125).

The isolation from mammalian tissue of a lipid inhibitor of blood clotting is further claimed by Chargaff (126, 127); the lipid was found associated with the cerebroside fraction obtained from sheep and pig brains, from the spinal cord of cattle, and from sheep red blood cells. Also the sphingomyelin fraction extracted by chloroform methyl alcohol from the spleen in a case of Niemann-Pick's disease contained a potent anticoagulant, which could be separated from the sphingomyelin by precipitation from ligroin solution with absolute alcohol (128). Purified sphingomyelin, prepared as described by Thannhauser & Setz, was entirely inactive in blood clotting (127), as was also pure cerebrin and kersin. The introduction of small amounts of sulfuric acid into these lipins resulted in the production of potent anticoagulants (129). In accordance with this the most active lipid fraction isolated from the Niemann-Pick spleen was found to contain appreciable amounts of sulfur. These interesting demonstrations of Chargaff are in harmony with the well established fact that sulfuric acid esters of polysaccharides act as strong anticoagulants.

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THE CHEMISTRY OF THE STEROLS

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During the three years which have elapsed since the chemistry of the steroids was last reviewed (1), so many important advances have been made in this field that it has been found necessary in these reviews to subdivide the subject into several sections. For this reason, and also through a limitation of space, we have confined our survey solely to the chemistry of the sterols, since a full appreciation of their reactions is a necessary prelude to a thorough understanding of sex- and cortical-hormone chemistry. Previous reviews in this series together with the monographs of Lettré & Inhoffen (2), Fieser (3) and Sobotka (4) provide an excellent background to the subject as a whole, while the articles by Strain (5) and Callow (6) describe the more recent advances. A more specialised treatment of the sterol field has recently been written by one of the authors in collaboration with Dr. F. S. Spring (7), and our aim in the present review has been to bring this up to date. The literature cited covers the period up to December, 1939, except that since the outbreak of hostilities, the German journals have not been available in this country.

Stereochemistry of the sterols.—This complex subject has not undergone any radical changes in the period under review and no solution of the difficult problem of the nomenclature of stereoisomerides which meets with universal approval has as yet been proposed. In order to obviate the changes necessitated by the proposal (8) to refer the orientation of the C_3 -hydroxyl group to that of the C_{10} -methyl group, Miescher & Fischer (9) now suggest making reference to the carbon atom in position 9, thus retaining the original "cis" and "trans" designations of Ruzicka, while eliminating the difficulty which arises when C_5 is attached to an ethenoid linkage.¹

¹ The system of indicating the location of the substituents above or below the plane of the ring system suggested by Linstead (13) has been adopted throughout this report, while the acid produced by cleavage of the ring system between C_x and C_y is represented as the $C_x \parallel C_y$ -dicarboxylic acid following the use of a similar convention by Sobotka (4). Trivial names have been employed wherever they are in general use or are more readily understood, the convention originally adopted by Fieser (3) being employed when necessary to denote stereochemical relationships.

From a consideration of the rates of hydrolysis of the acetates and benzoates of epimeric pairs of sterols, Ruzicka, Furter & Goldberg (10) find that in the compounds in which rings A and B are *trans*-oriented the derivatives are more readily saponified, conclusions borne out by the examination of models, in which steric hindrance is apparent in the *cis*-decalin orientation. Miescher & Fischer (11) studied the ease of formation of glucosides from similar epimeric pairs obtaining results concordant with those of Ruzicka except in the case of the two coprostanols, and to explain this anomaly they now conclude (9) that the influence of the substituent groups at C₅ and C₁₀ varies according to the nature of the reagent employed. Thus, C₁₀ orientation is responsible for glucoside and digitonide formation, while C₅ orientation appears to be the decisive factor in hydrolysis experiments. Bergmann (12) has drawn attention to certain cases in the steroid series where an absolute steric configuration can be defined, but since it is impossible to discuss the subject adequately in a review of this character the reader is referred to the original memoir.

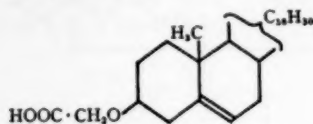
Steroid colour reactions.—A systematic study of the colour reactions of a number of sterols, bile acids and sex hormones with furfuraldehyde and sulphuric acid has been made (14). The position and number of ethenoid linkages present in the molecule are important factors, while the nature of the colouration produced appears to be affected by minor configurational changes of the functional groups. An analogous reaction employing benzaldehyde has also been investigated (15). Westphal (16) has collated the results obtained with the Tortelli-Jaffé reaction on various unsaturated sterols (cf. 17). The presence of a di-tertiary ethenoid linkage between two rings is usually necessary, the reactions given by ergosterol and similar compounds being explained by the ease with which migration takes place in such sterols to produce the 8:14 double bond. Other authors discuss steroid colour reactions (18, 19), a new reaction devised by Kägi & Miescher for the identification of steroids containing a 17-*cis*-hydroxyl group being also observed with sterols containing more than one ethenoid linkage, e.g., ergosterol and calciferol. von Christiani & Auger (20) have modified (by addition of lead tetraacetate) the Rosenheim trichloroacetic acid test for 7-dehydro-sterols. The modified test differentiates between the sterol and its esters and can be employed for the detection of amounts of such sterols of the order of 0.1 μ g.

Cholesterol derivatives.—Helferich & Gunther (21) have prepared the methylsulphonates of cholesterol, sitosterol and stigmasterol, from

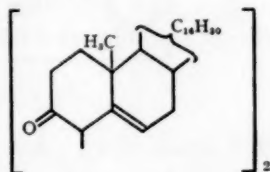
which steryl iodides are produced with sodium iodide in acetone. Cholesteryl iodide had previously been obtained by the action of hydrogen iodide on *i*-cholesteryl methyl ether (22).

In an attempt conveniently to prepare 7-hydroxycholesterol, the catalytic hydrogenation of 7-ketocholesterol derivatives has been further studied (23), but in neutral, as in acid media, preferential reduction of the ethenoid linkage is observed (cf. 24).

Water-soluble derivatives of steroids have been obtained by treatment with diazoacetic ester (25), cholesterol yielding, after hydrolysis, the acid I, the sodium salt of which is soluble.



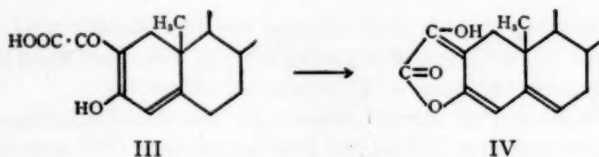
I



II

Irradiation of 3-keto- Δ^4 -cholestene (mercury arc or sunlight) produces a dimeric compound, probably $C_{54}H_{88}O_2$ (26, 27), and not $C_{42}H_{62}O_2$ as originally claimed (28), since on thermal decomposition it reproduces 3-keto- Δ^4 -cholestene. It shows no absorption in the ultraviolet above 2300Å and it is suggested that it has the constitution II, the non-reactive nature of the ketonic groups possibly being due to steric hindrance. Such a dimerisation appears to take place very readily with all Δ^4 -unsaturated steroid ketones, and may have some physiological significance since a number of the steroid hormones contain this grouping. If the irradiation is carried out in the presence of oxygen, 3,4-diketcholestane is formed (28).

Ruzicka & Plattner (29) have prepared 3-keto- Δ^4 -cholesteneoxalic acid, which exists in the enol form (III), by condensation of the ketone with ethyl oxalate and hydrolysis of the non-crystalline ester. This quantitatively regenerates the starting material on heating in a vacuum and is readily lactonized to IV with mineral acids in the

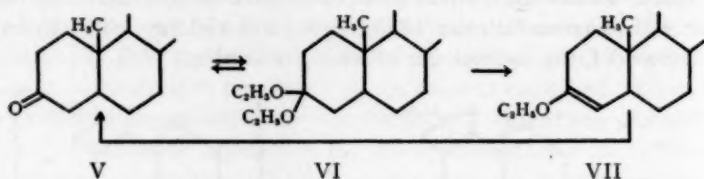


III

IV

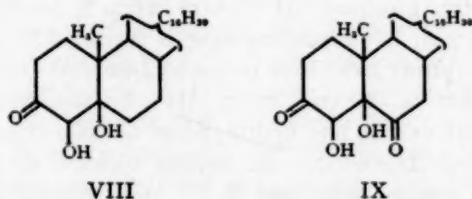
normal manner of these substances. Oxidation of the tetrahydro-lactone to the characteristic $C_2||C_3$ -dicarboxylic acid obtained by Windaus & Uibrig (30) from cholestanol proves the attachment of the oxalyl group in the 2-position.

3-Keto- Δ^4 -cholestene and other unsaturated steroid ketones readily yield enol ethyl ethers with ethyl orthoformate (31). With saturated ketones (32) such as cholestanone (V) it is possible to isolate the



diethyl acetal (VI), convertible in boiling xylene to the enol ethyl ether (VII). Cholestanone cyanohydrin has been prepared and on treatment with methylmagnesium iodide gives a mixture of 3-methyl- and 3-acetyl-cholestanols (33).

Oxidation of $\alpha:\beta$ -unsaturated ketones with perbenzoic acid or with hydrogen peroxide is difficult. Whereas 3-keto- Δ^4 -cholestene readily forms an oxide with perbenzoic acid the Δ^4 -isomer fails to react (34). Butenandt & Wolz (35) have shown, however, that addition can readily be brought about with hydrogen peroxide in the presence of osmium tetroxide, and by this means a number of diols, e.g., VIII and IX have been prepared from the corresponding unsaturated ketones.

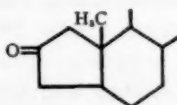


It is interesting to note that whereas the free sterols react readily, acetylation of the 3-hydroxyl group inhibits this reaction in the case of compounds containing a $\Delta^{5:6}$ -ethenoid linkage (36).

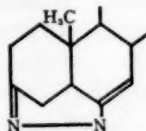
The reduction of steroid ketones by the Wolff-Kishner method has been investigated by Dutcher & Wintersteiner (37) who find that

steroids with a 3-keto group yield mainly the epimeric carbinols, while carbonyl groups in other positions in the steroid molecule are smoothly reduced to the corresponding hydrocarbons. If hydroxylamine is added the reaction with 3-ketones then proceeds normally. Reduction of 3-keto- Δ^4 -cholestene semicarbazone yields mainly Δ^4 -cholestene, β -cholestanol, α -coprostanol and possibly some *allo*cholesterol and cholesterol.

Δ^2 -Cholestene is converted to a 2,3-dihydroxycholestane with hydrogen peroxide (38), and the constitution of the diol has been established by its oxidation with chromic acid to the well known C_{27} -dicarboxylic acid. Pyrolysis of this acid (39) with acetic anhydride yields the ketone (X) which has been reduced to the epimeric



X

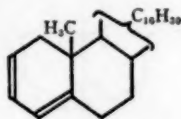


XI

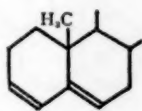
pyrocholestanols, which were separated with digitonin. The acetates of the latter have been oxidised to the corresponding pyroandrosterones.

Noller (40) has drawn attention to the necessity of determining the molecular weights of dihydropyridazine derivatives of 1,4-steroid diketones. Since he finds that the pyridazine (XI) obtained from 3,6-diketocholestane by treatment with hydrazine has a molecular weight nearly ten times as great as that expected for the simple compound, the mere preparation of such derivatives from supposed 1,4-diketones loses much of its diagnostic value.

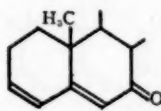
The cholestadienes.—Bergmann and his collaborators have undertaken a detailed examination (41) of the isomeric conjugated cholestadienes and have elaborated a method for the preparation of pure $\Delta^{2:4}$ -cholestadiene (XII) by dehydration of cholesterol with alumina, the isomeric $\Delta^{3:5}$ -cholestadiene (XIII) being obtained by Wolff-Kishner reduction of 7-keto- $\Delta^{3:5}$ -cholestadiene (XIV). Eck, von



XII



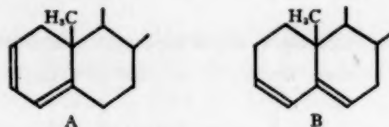
XIII



XIV

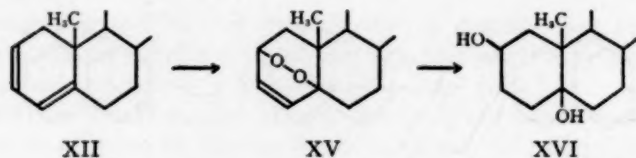
Peursem & Hollingsworth (42) prepared the $\Delta^{4:6}$ -diene from α - or β -cholestene dibromide with quinoline. It is now stated that pure $\Delta^{3:5}$ -cholestadiene has a rotation $[\alpha]_D$ of -123° , and for the $\Delta^{4:6}$ -isomer $[\alpha]_D$ is $+46^\circ$, while the highest recorded rotation for $\Delta^{2:4}$ -cholestadiene is $+168^\circ$. Undoubtedly many of the cholestadienes (cholesterilenes) recorded in the literature are mixtures of these three hydrocarbons.

From the results obtained in the study of the properties of the isomeric $\Delta^{2:4}$ - and $\Delta^{3:5}$ -cholestadienes and of ergosterol, Bergmann & Hirschmann (41) have formulated a number of rules which are of assistance in deciding whether a diene of the polycyclic series has a conjugated diethenoid system in one ring (type A) or divided between two rings (type B). Considerable differences are observed in the location of the ultraviolet absorption maxima (2650–2800 and 2300–2450Å respectively); addition of maleic anhydride is normal with A and such compounds are reduced with sodium and alcohol. Dienes of this type are readily isomerised by acids to the B dienes and are

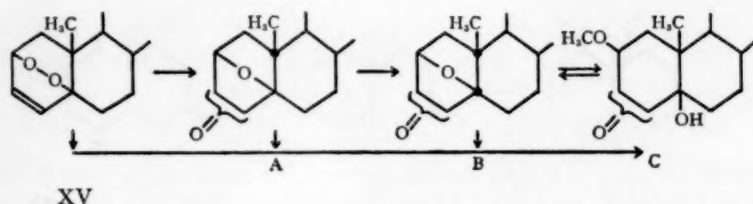


also photo-oxidised to the *trans*-annular peroxides mentioned below. It is pointed out that the rule observed by Callow & Young (43) concerning the influence of Δ^4 - and Δ^5 -ethenoid linkages on the optical rotation can also be usefully applied in the above cases. Ruzicka & Plattner (29) had already employed this rule to give an indication of the constitution of an unsaturated sterol lactone and Bergmann & Hirschmann (41), by applying the above rules, suggest that both 3-phenyl- and 3-chlorocholestadienes contain the $\Delta^{3:5}$ -diene system.

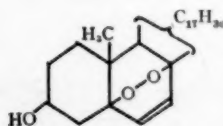
The constitution of the *trans*-annular peroxide (XV) formed on irradiation of $\Delta^{2:4}$ -cholestadiene (XII) with electric light has been established by hydrogenation to be a saturated diol (XVI); this is un-



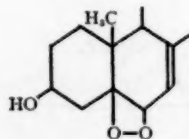
affected by lead tetraacetate, and yields only a monoacetyl derivative, reactions well represented by the following scheme. The original disagreement concerning the primary irradiation product (44) was explained by the quantitative isomerisation of the peroxide (XV) into a ketone, $C_{27}H_{44}O_2$ (ketone A), on insolation, also obtained directly from XII with sunlight. Ketone A is converted into a more stable isomeric ketone, B, either with acetic anhydride or on distillation; treatment of either isomer with methyl alcoholic potash yields another ketone C, also formed directly from the peroxide under the same conditions and reconverted into ketone B on distillation. A satisfactory explanation of these interconversions is represented below. Ketones A and B are considered by Bergmann, Hirschmann & Skau (41) to be stereoisomers differing in orientation around C_6 , the *trans*-



annular bridge being more stable in the *cis*-form (B). From the results of this study of the peroxide of cholestadiene, Bergmann deduces arguments in support of the contention of Fieser (3) that the constitution of ergosterol peroxide is better represented by a *trans*-annular formulation (XVII) than by the 5:6-peroxide formula (XVIII) originally suggested.



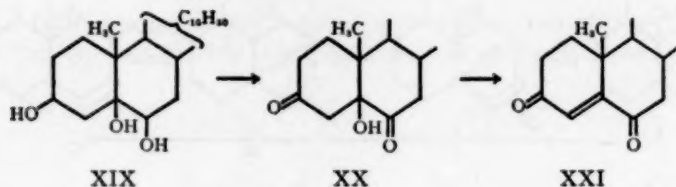
XVII



XVIII

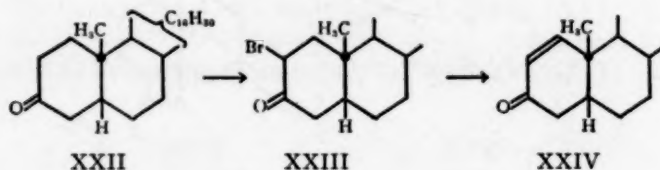
The cholestane-3,5,6-triols.—Two well-defined 3,5,6-triols have been prepared from cholesterol and its derivatives. Cholestane-3,5,6-

triol-I² can be prepared directly from cholesterol by treatment with hydrogen peroxide or alternatively by hydrolysis of α -cholesterol oxide (45, 46, 47, 48) and Criegee (49) has shown, employing reactivity with lead tetraacetate as the criterion, that the α -glycol grouping in this triol has the *trans*-configuration, while the *cis*-configuration is evident in cholestane-3,5,6-triol-II,² originally obtained by oxidation of cholesterol with permanganate (50) but recently prepared by a new method employing hydrogen peroxide and osmium tetroxide (51). In both triols I and II (XIX) the *cis*- or β -orientation of the 3-hydroxyl group to the C₁₀-methyl group persists and since on oxidation with chromium trioxide two isomeric hydroxy-diketones (XX) are produced, convertible into one and the same 3,6-diketo- Δ^4 -cholestene (XXI) by dehydration (50), it merely remains to establish the orientation of the 5-hydroxyl group, i.e., the *cis*- or *trans*-decalin nature



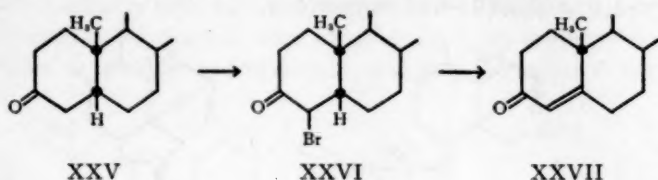
of the fusion of rings A and B in the two triols, in order to determine completely their constitutions.

Butenandt & Wolff (52) have clearly demonstrated that the position of entry of the bromine atom into 3-keto-saturated steroids is dependent on the *cis*- or *trans*-orientation of rings A and B. Cholestanone (XXII) and related steroids give 2-bromo-substitution products (XXIII), from which hydrogen bromide is eliminated only with difficulty (53) to yield Δ^1 -unsaturated ketones (XXIV) while com-

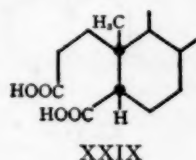
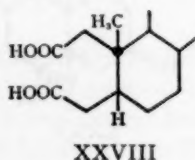


² The trivial name suggested for the triols (54) has been adopted to avoid confusion.

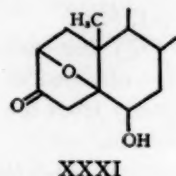
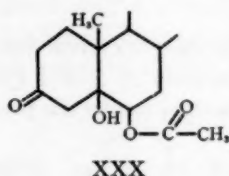
pounds of the coprostanone series (XXV) brominate in the 4-position (XXVI), the bromides readily yielding Δ^4 -unsaturated ketones (XXVII) with boiling pyridine. This behaviour is paralleled by the



oxidation of cholestanone to the $C_2||C_3$ -dicarboxylic acid (XXVIII), whereas coprostanone, on the other hand, yields the $C_3||C_4$ -dicarboxylic acid (XXIX).³ Ellis & Petrow (54) have studied both bromi-



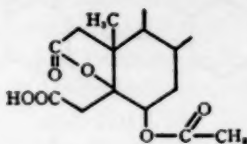
nation and oxidation of the hydroxy-ketone (XXX), prepared from the diacetyl derivative of cholestane-triol-I by partial hydrolysis and oxidation. They conclude that bromination involves substitution in the 2-position since hydrolysis of the monobromide with alcoholic potash produces a *trans*-annular oxide, the properties of which suggest that it may be represented by XXXI. Oxidation of the 6-acetyl



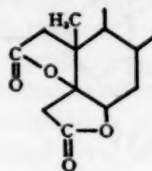
derivative of triol I gives XXX and in addition an acetoxylactonic acid (XXXII) which passes into a dilactone (XXXIII) on hydrolysis, the two latter products probably arising from an intermediate $C_2||C_3$ -dicarboxylic acid. On the assumption that the rules concerning the bromination and oxidation of 3-keto steroids are not invalidated when C_3 carries a hydroxyl group rather than a hydrogen atom, it is

³ See, however, Marker, R. E., *et al.*, *J. Am. Chem. Soc.*, **61**, 3317 (1939).

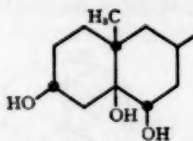
concluded that cholestane-triol-I has the *trans*-decalin configuration, and that the triols I and II can be represented by XXXIV and XXXV respectively, or described as cholestane-3(β),5(α),6(β)-triol and coprostane-3(β),5(β),6(β)-triol respectively.



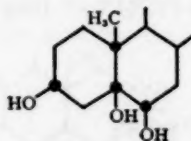
XXXII



XXXIII



XXXIV



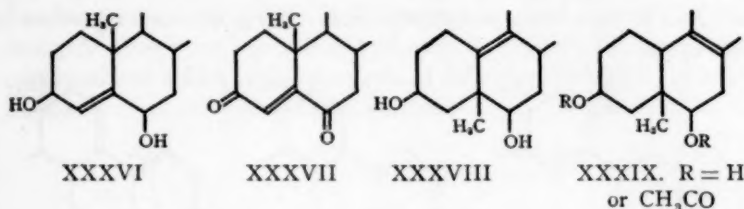
XXXV

The 3,5-dihydroxy-6-ketcholestane prepared by Heilbron, Jones & Spring (55), from the 5-bromo-ketone has been oxidised to the corresponding 5-hydroxy-3,6-dione, identical with that obtained from triol II (see above). If triol II is correctly represented as a coprostane derivative, then either the original bromination or alkaline hydrolysis must have been accompanied by a Walden inversion. Such rearrangements undoubtedly occur with great facility in this series of compounds (cf. 56).

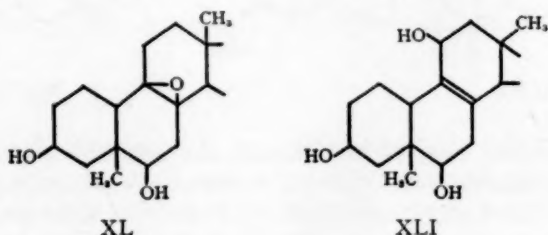
Hattori (57) has isolated 3,5,6-triacetoxycholestane from the mother liquors obtained in the preparation of triol I (46), and it is produced directly from the triol by acetylation in the presence of dry hydrogen chloride.

3,6-Dihydroxy cholestenes.—Dehydration of cholestane-3,5,6-triol-I with a sulphuric acid-acetic anhydride mixture (46, 47, 58) yields a diol, "Westphalen's diol," originally believed to be a 3,6-dihydroxy- Δ^4 -cholestene (XXXVI). Finding that the diol gave none of the expected 3,6-diketo- Δ^4 -cholestene (XXXVII) on oxidation, Lettré & Müller (59) suggested the interesting possibility that dehydration of the triol was accompanied by a retropinacolinic change and proposed XXXVIII as a possible constitution for the dehydrated product. [It has been suggested (60) that the elimination of hydrogen chloride

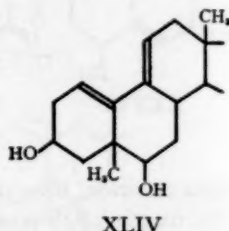
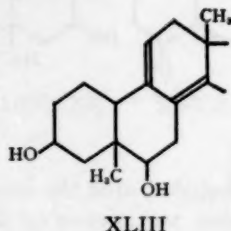
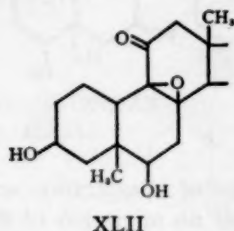
from 17-chloro-steroids is accompanied by a similar rearrangement.] Petrow, Rosenheim & Starling (61) examined this possibility after making a detailed examination of the properties of the diol. From its non-precipitability with digitonin, marked dextro-rotatory power, positive Tortelli-Jaffé reaction, ease of oxidation with selenium dioxide, resistance to catalytic hydrogenation, and non-isomerisation with hy-



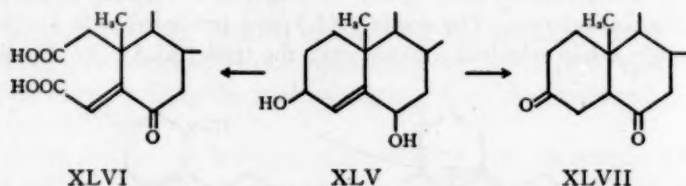
drogen chloride, they concluded, that the centre of unsaturation was in the inactive 8:9-position, and, assuming that no migration of the 3- and 6-hydroxyl groups had taken place, then "Westphalen's diol" is 5-methyl-3,6-dihydroxy- $\Delta^{8:9}$ -norcholestene (XXXIX, R = H). That the position assumed for the two hydroxyl groups by the latter authors is correct has been confirmed by Petrow (62) who obtained the diacetate of "Westphalen's diol" (XXXIX, R = Ac) by dehydration of the triol-diacetate with a sulphuric acid-propionic anhydride mixture, while the isolation of the C₂₅H₂₄ hydrocarbon on dehydrogenation (also obtained from cholesterol) provided proof that the methyl group attached originally to C₁₀ is still on a tertiary carbon atom. Oxidations with a variety of reagents are readily interpreted on this formulation. The oxide (XL) was produced with hydrogen peroxide, while selenium dioxide gave the triol (XLI), the 3,6-diacetate of which (the remaining hydroxyl group resisted acetylation) was oxidised with chromic anhydride to a keto-oxide (XLII) also



obtained directly from the original diacetate (XXXIX, $R = \text{Ac}$) by a similar method. Proof that none of these oxidations introduced substituents into the 7-position was available, since all the products were stable to lead tetraacetate, and hence were not α -glycols. Dehydration of the diacetates of both XL and XLI gave rise to a mixture of two dienes, which differed appreciably in the intensity of their absorption in the ultraviolet, and for which the constitutions XLIII and XLIV have been suggested, XLIII being the major product in both cases.

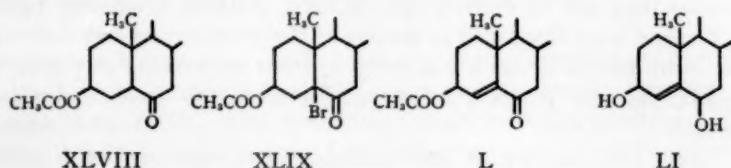


Oxidation of cholesteryl acetate with selenium dioxide gives the diacetate of an authentic 3,6-dihydroxy- Δ^4 -cholestene (XLV), originally believed (63) to be a *trans*-3,4-dihydroxy- Δ^5 -cholestene. Its constitution was conclusively proved by Butenandt & Hausmann (64) by oxidation both with chromic anhydride when the same $\text{C}_2||\text{C}_3$ -unsaturated ketodicarboxylic acid (XLVI) was obtained as from 3,6-diketo- Δ^4 -cholestene, and by the Oppenauer method to 3,6-diketocholestane (XLVII). The same diol (XLV) was also isolated (63)

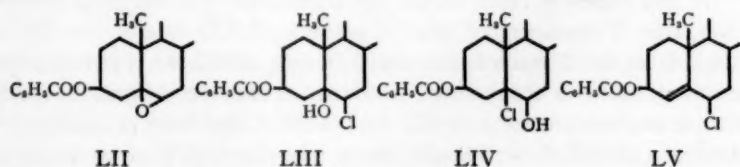


from the product obtained by treatment of cholesterol dibromide with sodium acetate, whereas cholesterol acetate dibromide with potassium acetate in alcohol or with silver nitrate in pyridine gives *cis*-3,4-dihydroxy- Δ^5 -cholestene (46), also formed along with XLV, by selenium dioxide oxidation of cholesteryl acetate (63, 64). Another 3,6-dihydroxy- Δ^4 -cholestene (LI), has been prepared by Heilbron, Jones &

Spring (55) by reduction of 6-keto-3-acetoxy- Δ^4 -cholestene (L), obtained from 6-ketocholestanyl acetate (XLVIII), via the 5-monobromide (XLIX). The constitution of L follows from its conversion with hot alcoholic potash into 3,6-diketocholestane (XLVII) and unless inversion at C₅ accompanied the aluminium isopropoxide reduction, this diol must differ from that of Butenandt & Hausmann (64) solely in the orientation of the hydroxyl group at C₆. Petrow, Rosenheim & Starling (61) have shown that the supposed "3,6-dihydroxy- Δ^4 -cholestene," prepared by Lettré & Müller (59) by quinoline treatment and subsequent hydrolysis of 3,6-dibenzoyloxy-5-chlorocholestane, is identical with 6-ketocholestanol.



Spring & Swain (65) describe the preparation of the two isomeric α - and β -cholesteryl benzoate oxides (LII), which are hydrolysed to the corresponding α - and β -cholesterol oxides (cf. 34). The α -benzoate oxide was converted into the chloro compound (LIII) by treatment with either hydrogen chloride or benzoyl chloride, the same compound being also obtained by the action of benzoyl chloride in pyridine on α -cholesterol oxide. Quinoline treatment effected reconversion into the α -benzoate oxide whereas thionyl chloride yielded the Δ^4 -chlorobenzoate (LV). With hydrogen chloride the β -oxide ben-



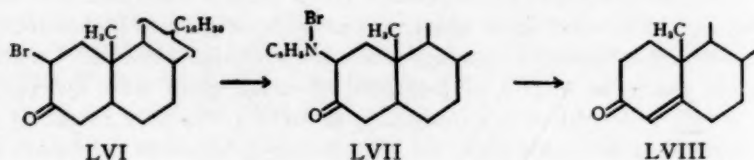
zoate gave an isomeric compound (LIV) readily converted into the known chloro-dibenzoate which was also obtained from β -cholesterol oxide or β -cholesteryl benzoate oxide with benzoyl chloride. On the other hand the acetate of β -cholesterol oxide gives with hydrogen chloride a derivative corresponding to LIII (34), thus revealing a remarkable difference from the corresponding benzoate. Ushakov &

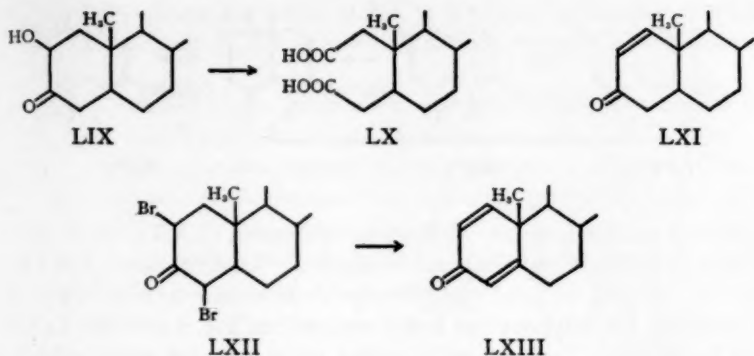
Madaeva (66) obtain 6-methylcholesterol and 3,5-dihydroxy-6-methylcholestanol by the action of methyl magnesium iodide on α -cholesterol oxide.

Bromination of sterol ketones.—The notable discovery of the synthetic estrogenic agents, e.g., stilboestrol (67), has rendered less attractive attempts to prepare the natural estrogens by aromaticisation and degradation of sterols. Such conversions, however, would certainly have an important theoretical significance in establishing constitutional relationships, while from the physiological aspect a convenient process for the preparation of estrone would probably be useful since clinical comparisons between it and the synthetic compounds may not be entirely satisfactory. Several promising routes to this end were developed in studies of the bromination and debromination of steroid ketones, but, judging from the number of published papers, interest in such investigations seems to have diminished recently.

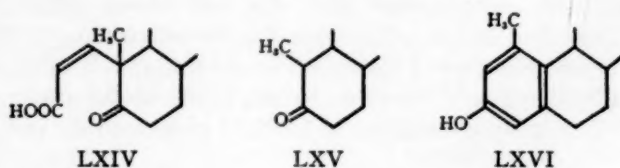
Ralls (68) has shown that cholestanone is converted with iodine monobromide into 2-bromocholestanone (LVI), which on further treatment with the same reagent gives a dibromocholestanone. By treatment of LVI with boiling pyridine Ruzicka, Plattner & Aeschbacher (69) have converted it into a pyridinium salt (LVII) which gives 3-keto- Δ^4 -cholestene (LVIII) on distillation; the same authors observed a similar reaction in the androsterone series. The constitution of LVI was conclusively established by its conversion into 2-acetoxycholestanone with potassium acetate in acetic acid and oxidation of the hydroxy compound (LIX) to the same $C_{22}||C_{25}$ -dicarboxylic acid (LX) as is obtained directly from cholestanol (30).

It has recently been shown by Butenandt and his collaborators (53), that Δ^1 -unsaturated steroid ketones (LXI) can be readily obtained from the 2-bromoketones with boiling collidine. It now appears, however, that the unsaturated ketones prepared from these bromides with potassium acetate in acetic acid at 200° , and hitherto believed to contain a Δ^1 -ethylene linkage, must now be regarded as being of unknown constitution. Either of these methods serves to convert



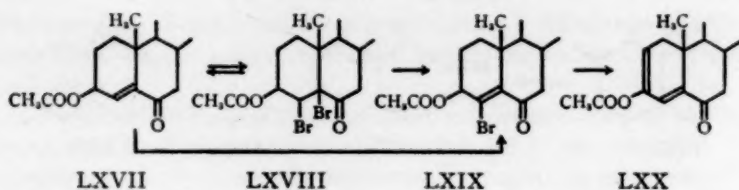


2,4-dibromocholestanone (LXII) into the doubly unsaturated ketone, 3-keto- $\Delta^{1:4}$ -cholestadiene (LXIII), originally prepared by Inhoffen & Huang-Minlon (70), who found that complete hydrogenation (palladium) yielded coprostanone, but in the presence of nickel, partial reduction gave 3-keto- Δ^1 -coprostene, while ozonolysis of LXIII yielded both the acid (LXIV) and the ketone (LXV), the dihydro-derivative of LXIV being identical with the acid obtained by the oxidation of 3-keto- Δ^4 -cholestene. The same authors (71) report that LXIII is rendered aromatic with acetic anhydride and sulphuric acid,

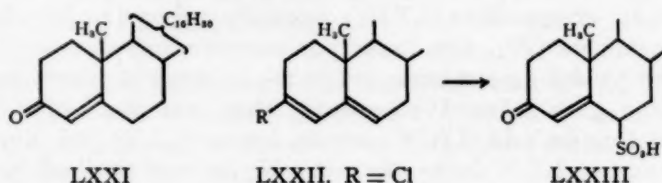


migration of the C_{10} methyl group, rather than its elimination as methane, producing the phenol LXVI, which readily couples with diazo salts.

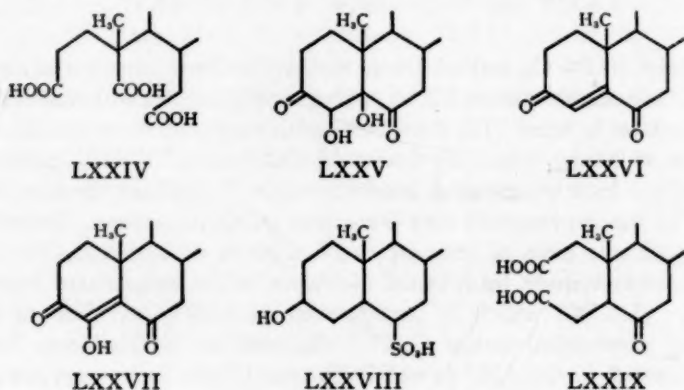
Jackson & Jones (72) found that with excess of bromine, the unsaturated ketone, 3-acetoxy-6-keto- Δ^4 -cholestene (LXVII), could be converted into a saturated keto-dibromide (LXVIII) from which LXVII was regenerated with potassium iodide in acetone. Bromination with one mole of bromine or elimination of hydrogen bromide from the dibromide, leads to the formation of the unsaturated bromo-ketone (LXIX) which is transformed in boiling pyridine to the doubly unsaturated ketone (LXX) also obtained by Heilbron, Jackson, Jones & Spring (56) from 5,7-dibromo-6-keto-cholestanyl acetate.



Steryl sulphonic acids.—3-Keto- Δ^4 -cholestene (LXXI) or its enol acetate (LXXII, R = OAc) and 3-chloro- $\Delta^{3:5}$ -cholestadiene (LXXII, R = Cl) all give in good yield 3-keto- Δ^4 -cholestene-6-sulphonic acid (LXXIII), the sulphonation being regarded as a 1:4 addition to the enol form (73). The acid and its alkali metal salts are water-soluble, and it is noteworthy that cholesterol, vitamin D, benzpyrene and

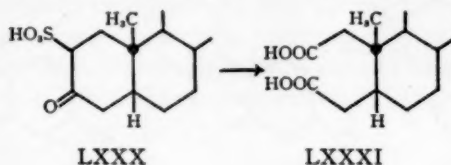


methyl cholanthrene are soluble in the aqueous solutions of the salts. Oxidation with permanganate gives the well known tribasic acid, LXXIV, together with 4,5-dihydroxy-3,6-diketcholestane (LXXV) [readily synthesised from 3,6-diketo- Δ^4 -cholestene (LXXVI)], which can be dehydrated to a triketone, which exists in the enolic form (LXXVII). Catalytic reduction of LXXIII gives both di- and tetra-

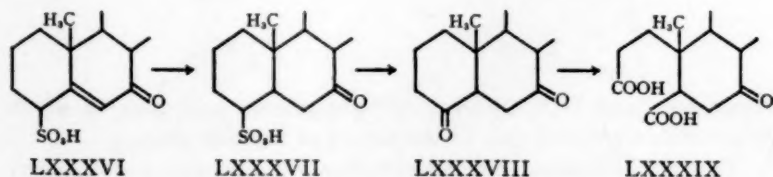
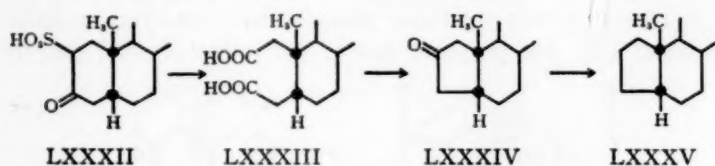


hydro-derivatives, the latter (LXXVIII) being oxidised to the keto-dicarboxylic acid (LXXIX), which is also obtained from 3,6-diketo-cholestane.

Sulphonation of cholestanone gives cholestanone-2-sulphonic acid (LXXX) which yields the $C_2||C_3$ -dicarboxylic acid (LXXXI) on oxidation, while coprostanone surprisingly provides a mixture of the 2- and 4-sulphonic acids, the latter on oxidation giving the $C_3||C_4$ -dicar-



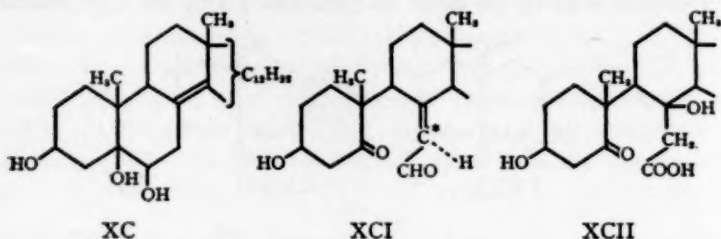
boxylic acid, also obtained from coprostanol. The acid (LXXXIII) obtained by oxidation of the 2-isomer (LXXXII) was not previously known, but on cyclisation it gave a ketone (LXXXIV) and reduction yielded the corresponding hydrocarbon (LXXXV) which had been prepared by Lettré (74). A 4-sulphonic acid (LXXXVI) is the product from 7-keto- Δ^5 -cholestene and oxidation of the dihydro acid (LXXXVII) gives 4,7-diketocholestane (LXXXVIII) and the known keto-dicarboxylic acid (LXXXIX), obtained from Diels' acid. 3,6-



Diketo- Δ^4 -cholestene forms a 2-sulphonic acid, its constitution being proved by oxidation of the dihydro acid (also obtained by direct sulphonation of the saturated diketone) to the keto-dicarboxylic acid (LXXIX). $\Delta^3:6$ -Cholestadiene gives a 6-sulphonic acid, oxidation of

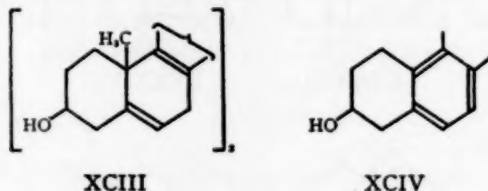
the lithium salts of the dihydro acid to 6-ketocholestane, and of the parent acid to 4,5-dihydroxy-3,6-diketocholestane, proving the position of entry of the sulphonic group.

Ergosterol and 7-dehydro-sterols.—Huang-Minlon (75) has further investigated the products obtained by the action of lead tetraacetate on trihydroxy-ergostadiene (XC). In addition to the $\alpha:\beta$ -un-



saturated keto-aldehyde (XCI) isolated by Heilbron, Morrison & Simpson (76), a stereoisomer (*) has been obtained, its constitution being proved by oxidation with hydrogen peroxide to the same keto acid (XCII) as is obtained from the original keto-aldehyde.

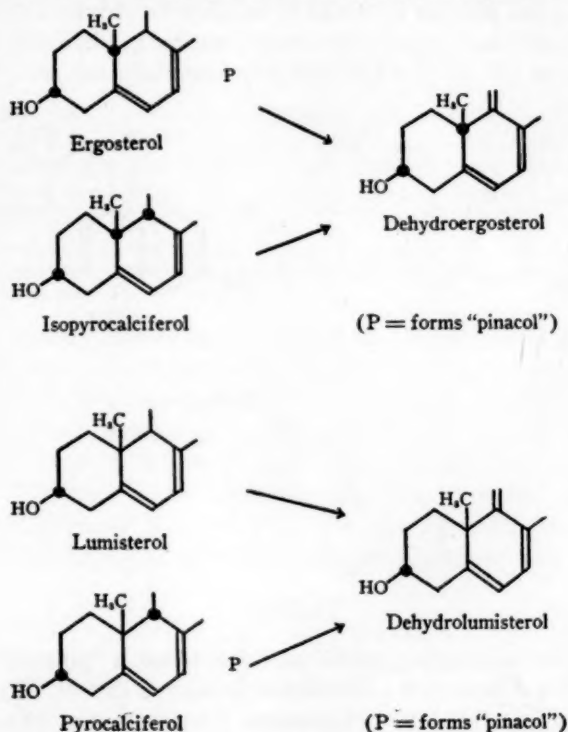
Ando (77) has demonstrated that the formation of high melting "pinacols" from ergosterol and 7-dehydrocholesterol is not inhibited if the irradiation is carried out under modified conditions and Inhofen (78) has suggested that these "pinacols" have the structure XCIII. Haslewood (79) has prepared similar derivatives from 7-dehydro-



stigmasterol and 3-(β)-hydroxy- $\Delta^{5,7}$ -choladienic acid, both of which differ from ergosterol only in the nature of the side chain.

Further interesting interrelationships between the stereoisomers ergosterol, lumisterol, pyrocalciferol and isopyrocalciferol have been revealed by the observation of Kennedy & Spring (80) that pyrocalciferol acetate on insolation, yields a "pinacol" diacetate which, like the "pinacol" diacetate of ergosterol, loses methane on vacuum distillation to give neoergosteryl acetate. Isopyrocalciferol acetate and

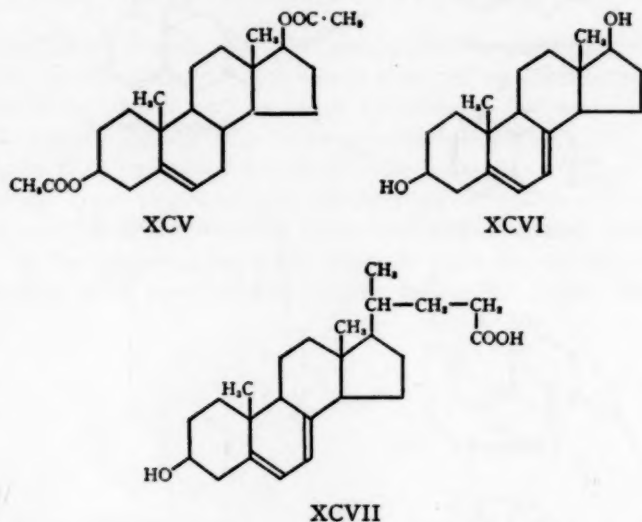
lumisterol are unaffected by prolonged insolation, and it appears that the $C_9:C_{10}$ -orientation is the decisive factor since dehydroergosterol and dehydrolumisteryl acetate, in which the asymmetry around C_9 no longer exists, both yield "pinacols." The fact that neoergosterol (XCIV) is isolated on pyrolysis of the pyrocalciferol "pinacol" suggests that epimerisation of the C_3 -hydroxyl group does not take place either during the irradiation of ergosterol to yield lumisterol or during the pyrolysis of calciferol, and that the four stereoisomers do not differ in this respect. It is thus possible to arrive at the appended structures for these four compounds, assuming that the C_3 -hydroxyl group and the C_{10} -methyl group are *cis*- or β - in ergosterol.



In addition to neoergosterol, Urushibara & Ando (81) have isolated "isoneoergosterol" from the pyrolysis product of ergosterol "pinacol" and Ando (77) has recently shown that this is in fact a molecular complex of neoergosterol with a new sterol derivative, iso-

dehydroergosterol ($C_{28}H_{42}O$), different from dehydroergosterol, although it forms an insoluble digitonide.

An interesting analogue of 7-dehydrocholesterol has been prepared by Butenandt, Hausmann & Paland (82) who, starting with the diacetate of 3,17-dihydroxy- Δ^5 -androstene (XCV), followed the route employed by Windaus, Lettré & Schenck (83) in preparing 7-dehydrocholesterol itself. The 3,17-dihydroxy- $\Delta^{5:7}$ -androstadiene (XCVI) so obtained, exhibits similar absorption in the ultraviolet to that of ergosterol and allied substances, insolation in the presence of eosin gives a peroxide, $C_{28}H_{44}O_4$ (84) as with 7-dehydrocholesterol, and irradiation (with light from a mercury arc) parallels that of ergosterol except that the product is devoid of antirachitic activity (85). Haslewood (79, 86) has prepared by similar methods, 3-hydroxy- $\Delta^{5:7}$ -choleladienic acid (XCVII) which exhibits essentially the same light ab-



sorption properties as ergosterol and also forms a "pinacol," but the antirachitic potency of the irradiation product is minute. Elimination of benzoic acid from the intermediate dibenzoate was effected with boiling dimethylaniline, a process which is an improvement on the pyrolytic procedure (83) previously used to convert the dibenzoate of 3,7-dihydroxy- Δ^5 -cholestene into 7-dehydrocholesterol.

Since Wunderlich (87) has shown that 7-dehydrositosterol has some provitamin-D activity, it seems remarkable that the stigmasterol

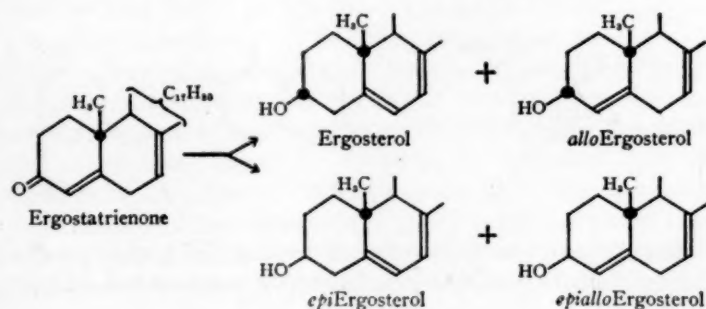
analogue prepared by Linsert (88), should exhibit no such properties. Haslewood (79) has now confirmed this finding; this specificity in provitamin-D activity is mysterious, especially since neither conversion of the Δ^{22} -ethenoid linkage of ergosterol into the oxide ring nor epimerization of the 3-hydroxyl group appear to have any deleterious influence on the biological activity of the irradiation products (85). It is also interesting to note that *epilumisterol* (7) and the pyrocalciferols, all of which possess the ergosterol unsaturated system in ring B, are unable to function as provitamins.

TABLE I*
PROVITAMIN-D ACTIVITIES OF STEROLS

Positive	Negative
Ergosterol	<i>epi</i> Lumisterol (7)
Lumisterol	Pyrocalciferol (90)
<i>epi</i> Ergosterol (85)	Isopyrocalciferol (90)
Ergosterol-22-oxide (85)	7-Dehydrostigmasterol (88)
22-Dihydroergosterol (89)	3-Hydroxy- $\Delta^{5:7}$ -choladienic acid (79)
7-Dehydrocholesterol (83)	3,17-Dihydroxy- $\Delta^{5:7}$ -androstadiene (85)
7-Dehydrositosterol (87)	

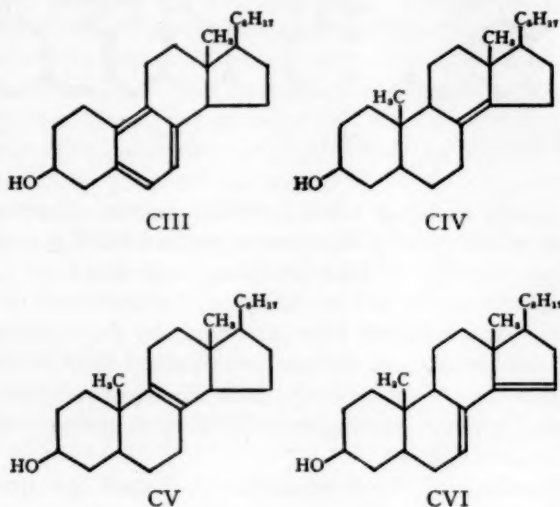
* *epi*-7-Dehydrocholesterol has been prepared from *epicholesterol*, and after irradiation is one-tenth as biologically active as 7-dehydrocholesterol similarly irradiated [Windaus, A., and Naggatz, J., *Ann.*, 542, 204 (1939)].

The vexed question of the nature of the products obtained on reduction of ergostatrienone (ergosterone) now seems to be satisfactorily settled. Windaus & Buchholz (91) recently record the isolation of *allo*ergosterol and traces of *epi*ergosterol from the reduction product, in addition to the ergosterol and *epiallo*ergosterol already described (92, 93). These findings have been confirmed in part by Epprecht, Heilbron & Jones (94). As was expected, *allo*ergosterol was found to be more easily dehydrated to a tetraene than ergosterol



col," smoothly convertible into the nor-sterol (CIII), while on catalytic reduction both CI and CII give primarily α -cholestenol (CIV) and

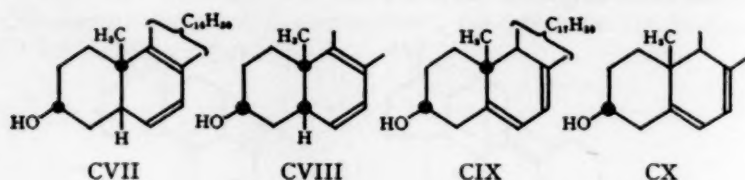
finally cholestanol, thus disposing of the possibility that they might be stereoisomeric. On reduction with sodium and propyl alcohol, δ -cholestenol (probably CV) is formed, its acetate being isomerised



(palladium-hydrogen) into the known α -cholestenol (CIV). Both isodehydrocholesterol and 7-dehydrocholesterol undergo isomerisation with hydrogen chloride to cholestadienol-B₈ (CVI).

Considerable progress has been made in the study of the photochemical changes induced on irradiation of sterols with a conjugated diethenoid system in ring B, and it has been found that stereochemical differences have a powerful influence on the mechanism of the irradiation. The initial steric-inversion at C₁₀ of ergosterol into lumisterol, followed by ring fission to tachysterol and finally calciferol has now been definitely established. With the pyrocalciferols, however, Dimroth (98) observed an entirely different reversible transformation which quantitatively produced compounds, containing no conjugated unsaturated system, from which the pyro-sterols were reproduced on heating. Continuing this study, Windaus & Zühlendorf (99) have now examined the primary irradiation product of isodehydrocholesterol (3-hydroxy- $\Delta^{6:8}$ -cholestadiene) (CVII). They obtained a compound very closely analogous to the starting material, both in absorption spectrum and chemical properties. It differed mainly in the fact

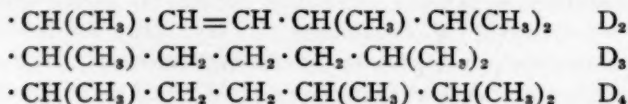
that complete hydrogenation produced coprostanol, rather than cholesterol, and its reactions all point to a structure CVIII; this photo-



chemical inversion being exactly similar to that observed in the production of lumisterol (CX) from ergosterol (CIX), except that a hydrogen atom, instead of a methyl group, is involved.

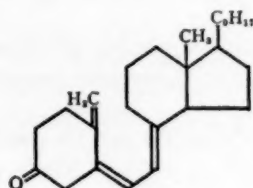
Further additions to our knowledge of the behaviour of the 7-dehydrosterols on irradiation have been made by the Göttingen school in detailed examinations of the products obtained from 7-dehydrocholesterol (100) and, more recently, from 22-dihydroergosterol (101). In both cases products analogous to lumisterol, tachysterol and calciferol have been isolated.

The chemistry of the D-vitamins.—Attention has already been drawn to the provitamin-D activity of various dehydrosterols, but owing to practical difficulties, from only three of these, ergosterol, 7-dehydrocholesterol and 22-dihydroergosterol, have the crystalline, biologically active fractions of the irradiation products, vitamins D₂, D₃ and D₄, respectively, been obtained. The chemistry of these vitamins has been studied largely by Windaus and his collaborators and an excellent summary has been compiled by Brockmann (102). A discussion of the biological aspects is beyond the scope of this review, but it is perhaps not out of place to draw attention to the fact that slight changes in the side chains of the vitamins are responsible for marked differences in the antirachitic activity when tests are carried out on rats and chicks. Whereas vitamins D₂ and D₃ are equally potent in curing rachitis in rats, the former is barely half as active as D₃ for chick rachitis and D₄, only half as active as D₂ or D₃ with rats, is more active than D₂ with chicks. The slight, but apparently significant variations in the side chains of the three vitamins are illustrated here:

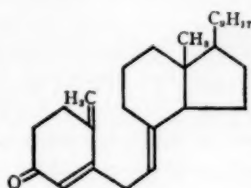


Brockmann & Busse (103) have isolated small quantities of vitamin D₂ (calciferol) from fish liver oils, of which vitamin D₃ (synthetically obtained from the irradiation products of 7-dehydrocholesterol) is the chief antirachitic component. This, we believe, is the first recorded isolation of vitamin D₂ from natural sources.

Calciferol has been oxidised to a non-crystalline ketone (104) by the Oppenauer method. This ketone, which has only a very low antirachitic potency, gives poor yields of calciferol by the reverse reduction process. The semicarbazone, from which the original ketone can be regenerated by benzaldehyde treatment, yields an isomeric ketone on decomposition with oxalic acid in the normal manner, doubtless due to the labile nature of the unsaturated system present in the original ketone, for which the formulation CXI or CXII is suggested.

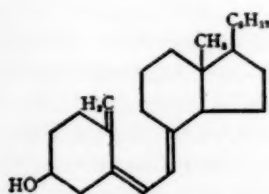


CXI

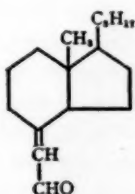


CXII

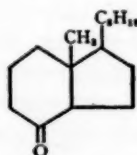
Formaldehyde can be detected amongst the products of the ozonolysis of vitamin D₃ (CXIII), and the isolation of an $\alpha:\beta$ -unsaturated aldehyde (CXIV) and the saturated ketone (CXV), similar to those obtained by the ozonolysis of calciferol, is reported (103); these results provide further evidence of the correctness of the structure suggested for the vitamin.



CXIII



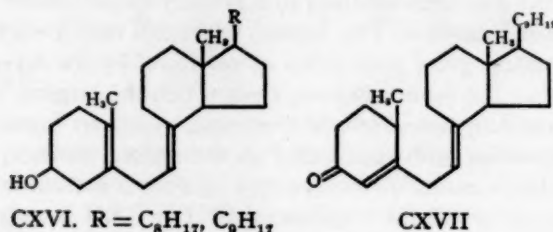
CXIV



CXV

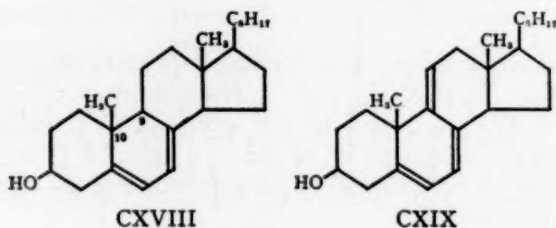
The same saturated ketone (CXV) is obtained (105) by oxidation with ozone of the allophanate of dihydrovitamin D₃ (prepared by

reduction of the vitamin with sodium and propyl alcohol) proving the presence of a 7:8-, and eliminating the possibility of an 8:14-ethylenic linkage in the dihydro derivative, the constitution of which may be represented by CXVI ($R = C_8H_{17}$). Dihydrovitamin D_2 , prepared



by a similar reduction, evidently has a similar structure (CXVI) ($R = C_9H_{17}$), since oxidation with the Oppenauer reagent gives the α : β -unsaturated ketone (CXVII).

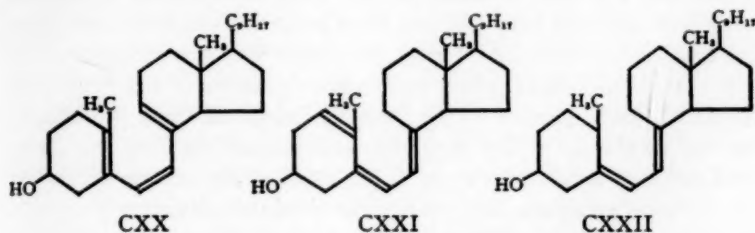
Further similarities between vitamins D_2 and D_3 are revealed by the production of pyro- and isopyrovitamins D_3 (106) by pyrolysis of



the vitamin, and these, along with 7-dehydrocholesterol and lumisterol-3 are closely analogous in properties and interrelationships to the corresponding members of the ergosterol series (p. 155). The pyro and isopyro compounds could not be obtained crystalline but dehydrogenation (mercuric acetate) of crystalline derivatives yielded two dehydro derivatives (CXIX) respectively identical with those obtained by similar oxidations of lumisterol-3 and 7-dehydrocholesterol. Assuming a *trans*- C_9 : C_{10} - configuration for 7-dehydrocholesterol the relationships between the four stereoisomers (CXVIII) can be conveniently expressed thus:

	C ₁₀	C ₉		C ₁₀
Lumisterol-3	+	+	Dehydro-	
Pyrovitamin D ₃	+	—	lumisterol-3	+
Isopyrovitamin D ₃	—	—	Tetradehydro-	
Dehydrocholesterol	—	+	cholesterol	—

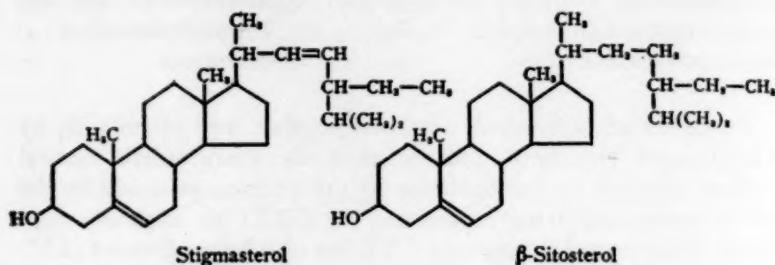
Oxidation of tachysterol, produced together with vitamin D₂ by irradiation of ergosterol, fails to yield the characteristic neutral products obtained by the oxidation of the vitamin, as would be the case if tachysterol were represented by CXXI as originally suggested. The alternative structure CXX has now been advanced (107) in agreement with the observation that acidic products are mainly obtained, even by ozonolysis of the crystalline tachysterol adduct with the dimethyl ester of acetylene dicarboxylic acid. Von Werder (108) has made a further study of the products produced on reduction of tachysterol with sodium and alcohol and has isolated both dihydrovitamin D₂ (109) and dihydrotachysterol (CXXII). The latter gives the same saturated ketone as is obtained from calciferol on oxidation



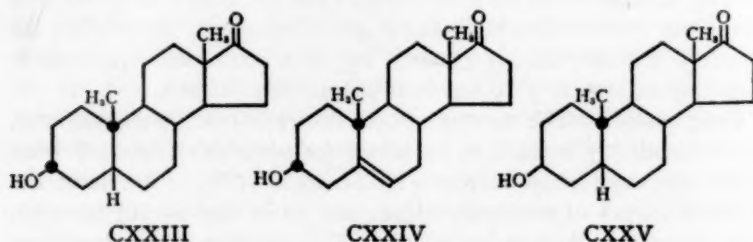
with chromic anhydride (110), indicating the presence of a 7:8-ethenoid linkage, while its absorption in the ultraviolet indicates that two of the three ethenoid linkages are conjugated. It exhibits an extremely low antirachitic potency but is a most active calcinosis-producing substance, a 10 µg dose being toxic to mice.

Other sterols.—The structure of the diethenoid sterol, stigmasterol, most conveniently isolated as its acetate-tetrabromide from soy bean oil, has been completely elucidated by Fernholz (111). Fucosterol, the diethenoid sterol of numerous algae, and an isomer of stigmasterol, yields stigmastanol on hydrogenation (112) as does the isomeric ostreasterol from oysters (113). On the other hand, the doubly unsaturated sterol from rape seed oil, brassicasterol, has also been shown

to be isomeric with stigmasterol, but the properties of brassicasterol differ from those of stigmasterol (114).⁴



It was first demonstrated by Anderson and his collaborators (115) that the common plant sterol, sitosterol, was not homogeneous. The sterols isolated from various sources exhibited marked differences in properties, attributed by those workers to the presence, in varying proportions, of three isomeric sitosterols ($C_{29}H_{50}O$) designated α -, β -, and γ -sitosterols. γ -Sitosterol, the most insoluble constituent of the mixture, was readily obtained pure from the sterol of maize and wheat germ oils and later workers have isolated β -sitosterol in a pure state from cotton seed oil (116). An insight into the constitution of the β - and γ -sterols was gained by oxidative removal of the side chain. Ruzicka & Eichenberger (117) isolated isoandrosterone (CXXIII) from the oxidation of the acetyldihydrositosterol (mainly γ -) from wheat germ oil while Oppenauer (118) oxidised the acetate dichloride of γ -sitosterol from soy bean oil and obtained dehydroisoandrosterone (CXXIV). Similar degradation of β -sitosterol, after conversion into the acetate of the *epi*-dihydro-sterol, yielded androsterone (CXXV) (119). These experiments provide proof that cholesterol, β - and γ -



⁴ See, however, Fernholz, E., and Stavelly, H. E., *J. Am. Chem. Soc.*, **62**, 428 (1940).

sitosterol have the same ring structure and substituents in the same positions, and that the two latter differ only in the nature of the side chains.

The identity of β -sitosterol with 22-dihydrostigmasterol, and of the fully hydrogenated sterols, has now been firmly established by a number of workers (120, 121, 122). Bonstedt (123) has observed a marked depression in melting point on mixing γ -sitostane and stigmastane, but evidence as to the precise nature of the difference is not yet available. It is interesting to record that cinchol, the sterol of cinchona bark, is now considered to be identical with β -sitosterol (124).

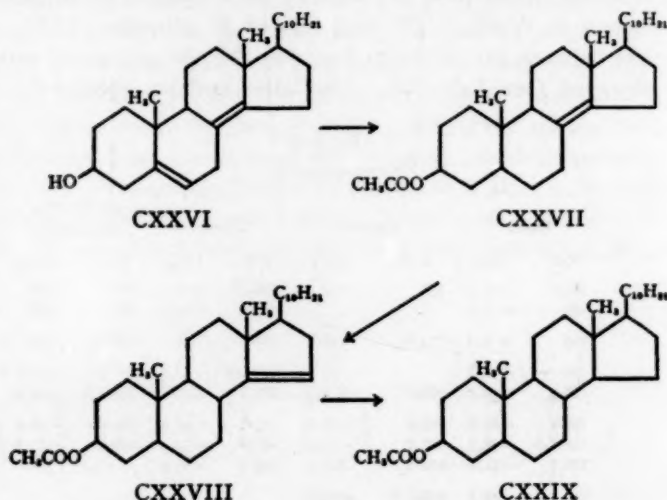
The constitutions of β - and γ -sitosterols having been determined attention was next directed towards other constituents of the sitosterol complex, more especially towards the more soluble α -sitosterol fraction. Wallis & Fernholz (125) examined this fraction from wheat germ oil, and after removal of some β -sitosterol as the benzoate, fractionated the dinitrobenzoates and achieved the separation of α_1 - and α_2 -sitosterols. Both of these were precipitable with digitonin and possessed two ethylenic linkages, and the analytical data suggested that while the former was isomeric with stigmasterol the latter was possibly a homologue, $C_{30}H_{50}O$. Another constituent, α_3 -sitosterol, of the α -sitosterol complex has recently been isolated simultaneously by Bernstein & Wallis (126) and Gloyer & Schuette (127), good agreement between the constants found by the two groups of workers being observed (see Table II). The latter authors isolated the new

TABLE II
SITOSTEROLS

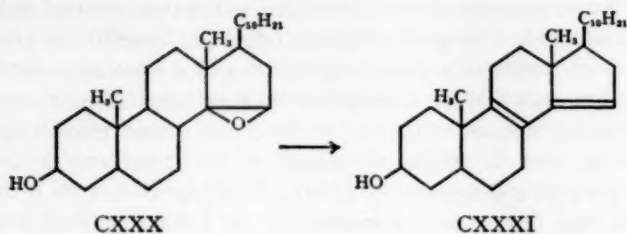
	Sterol		Acetate		Benzoate		3,5-Dinitrobenzoate	
	m.p.	$[\alpha]_D$	m.p.	$[\alpha]_D$	m.p.	$[\alpha]_D$	m.p.	$[\alpha]_D$
α_1	164-6	-1.7	187	+29	168-72	+42	222	+37 (126)
	163	-1.8			167-8	+40.8	225	+37 (127)
α_2	156	+3.5	124-6	+17	164-6	+27	206	+26 (125)
α_3	142	+1.7			167.5-8	+14.9	202.5-3	+15.4 (127)
	143-3	+5.2	153-3	+6.1	173-5	+12.0	210-1.5	+12.2 (126)
	136-7	-36.6	125-6	-41.0	146-7	-13.8	202-3	-10.4 (116)
β	135-5.5	-34.2	126-7	-34.7	145-6	-14.2	207-9	-21.7 (128)
	136-7	-31.5	123-3	-36.6	146-7	-12.2		(129)
γ	146	-42.0	143-3.5	-45.0				(127)
	147	-42.8	143-3.5	-45.8	152	-19.6		(129)
δ	136-7	-23.9	113.5-4.5	-24.4	157-8	-16.0		(129)
ϵ	143-4	-38.7	127-8	-44.7			215-7	(128)

sterol along with α_1 -, β -, and γ -sitosterols from rye germ oil, while Bernstein & Wallis isolated the α_1 -, α_2 -, and α_3 -sterols from the more soluble sitosterol fraction of wheat germ oil. This new sterol appears to be yet another isomer of stigmasterol and it has been observed that the Salkowski reaction of these α -sitosterols resembles very closely that of ergosterol. During a study of the constituents of sarsaparilla root, Simpson & Williams (128) have separated a sterol which is probably identical with β -sitosterol together with a small quantity of a new ϵ -sitosterol, $C_{29}H_{50}O$, while Ichiba (129) records the isolation of a δ -sitosterol.

More recently, Bernstein & Wallis (130) describe experiments directed toward the elucidation of the structure of α_1 -sitosterol, for which the constitution CXXVI has now been suggested. Catalytic hydrogenation of the acetate of CXXVI gave only a dihydro compound (CXXVII), readily isomerised by hydrogen chloride to an isodihydro-acetate (CXXVIII) which could then be reduced further to the saturated α_1 -sitostanyl-acetate (CXXIX). It is pointed out that these latter changes are very reminiscent of those undergone by α -ergosterol (131) and α -cholestenol (132) and since the saturated sterol differs from stigmastanol, it is concluded that this difference is solely



due to side chain isomerism. The isolation of a conjugated diene (CXXXI) by acid hydrolysis of the oxide of α_1 -isodihydro-sitosterol



(CXXX) provides further proof of the correctness of the suggested structures since a similar behaviour has been observed with β -ergosterol oxide (133). While reasonable proof of the position of the inert ethylenic linkage in α_1 -sitosterol has been provided, the location of the readily reducible linkage has been assumed by analogy, since "all natural sterols which are unsaturated have been found to have one double bond at the 5:6-position." It has, however, been observed in this laboratory (unpublished results) that zymosterol, an important diethenoid constituent of the yeast sterol complex, does not contain such an ethylenic linkage, and, as mentioned below, there is reason for believing that this is true of other naturally occurring sterols.

Isomeric with stigmasterol, and closely related in properties to the α -sitosterols, are the three spinasterols, isolated from spinach fat (134). α -Spinasterol is readily isolated from senega root (135) and Fernholz & Moore (136) have identified it as a constituent of the non-saponifiable portion of alfalfa meal, a finding supported by the study of King & Ball (137) on alfalfa seed oil. It accompanies vitamin K in the alfalfa extracts and the medicagosterol II isolated by Karrer (138) is probably also α -spinasterol. The three spinasterols all yield spinastanol on hydrogenation and Larsen (139) has proved its identity with stigmastanol. The two ethenoid linkages of α -spinasterol are not conjugated, neither is there unsaturation in the side chain, and one of the nuclear double bonds is inert, as was found with α_1 -sitosterol. In view of the low laevo-rotation of the sterol, and the fact that α -spinastadienone is not an α : β -unsaturated ketone, Simpson (135) has suggested that the familiar 5:6-ethylenic linkage is absent from this sterol.

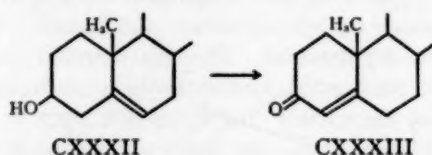
The non-saponifiable portion of sugar-cane wax has been examined by Mitui (140) and in addition to stigmasterol and (probably) β -sitosterol a saturated diol, α -saccharostanediol, $C_{29}H_{52}O_2$, and an unsaturated ketone, β -saccharostenone, $C_{29}H_{48}O$, have been isolated.

The dextrorotatory tritisterols and orysterols, isolated from wheat germ oil (141, 142) and rice germ oil (143) respectively are precipitable with digitonin and may represent a new class of natural sterols. They are regarded as having the composition $C_{30}H_{50}O$ and this, as also their abnormal behaviour in the sterol colour reactions, suggests that they may be related to certain of the triterpene resinols, such as lanosterol and agnosterol (144). Small quantities of α -theosterol, which may belong to the same class have been isolated from cacao germ oil (145).

β -Equistanol (146), isolated from the urine of pregnant mares and other mammals, is only found in animal urine, a fact which may have some physiological significance. This saturated sterol, which is precipitable with digitonin, appears to have the composition $C_{30}H_{54}O$, and Marker (148) has suggested that it may be identical with dihydro- α -tritisterol (141). Butenandt & Dannenbaum (147) reported the isolation of cholesterol from human pregnancy urine and during the course of a systematic survey Marker (148) has also recently shown it to be present in animal urine.

General reactions.—A novel method of separation of sterol mixtures has been employed by Ladenburg, Fernholz & Wallis (149) who have chromatographically resolved an artificial mixture of cholesterol, stigmasterol and ergosterol by means of their coloured azobenzene-4-carboxylates.

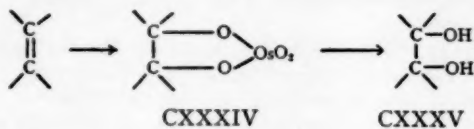
Oxidation using the Oppenauer method (150) (with aluminium *tert.*-butoxide and an excess of acetone in a neutral solvent) has provided a convenient method for the conversion of Δ^5 -unsaturated sterols (CXXXII) into the Δ^4 -unsaturated ketones, and the reaction can



be utilised as a test for the presence of a Δ^5 -ethenoid linkage in a 3-hydroxy sterol. A modification of this method, which employs the more readily accessible aluminium isopropoxide at a higher temperature has been fully exploited in the steroid series (151).

Criegee (152) suggested the use of osmium tetroxide for the conversion of cyclic unsaturated compounds into *cis*- α -glycols (CXXXV),

the intermediate osmic ester (CXXXIV) readily decomposing with sodium sulphite. The yields obtained with this process are uniformly high and the reaction is being used to a considerable extent in steroid chemistry (153), certain applications having already received mention in this report.



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CHEMISTRY OF AMINO ACIDS AND PROTEINS

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The work of S. P. L. Sørensen, who died early in 1939 [biographical notices by Linderstrøm-Lang (1, 2) and Cohn (1)], is recognized as fundamental for all contemporary progress in this field. His clarification of protein chemistry, by the painstaking application of the quantitative methods of analytical and physical chemistry, was a scientific achievement of enduring significance.

The current widespread interest in this branch of chemistry is reflected by the number of symposia which have been reported during the year.¹ These symposia, together with the recent books by Schmidt and by Lloyd & Shore, and the reviews by Bergmann and by Cohn (2), may be consulted for an account of the present state of knowledge of amino acid and protein chemistry.

The present article is not intended to be a comprehensive summary of the field or of any part of it, but merely a guide to the reading of recent papers. The period covered is essentially that included in *Chemical Abstracts*, 33 (1939). Each of the papers cited has been consulted in the original (with the exception of the German symposium of the preceding list). Some citations have been omitted because the journals were not available to the reviewer, and many because of the necessary limitation of space.

AMINO ACIDS AND PEPTIDES

Preparation and synthesis.—Redemann & Dunn obtained good yields of seven common amino acids by a synthesis involving ethyl

¹ Chemistry and physiology of proteins, 3. *Frankfurt. Konf. med.-naturw. Zusammenarbeit*, 203 pp. (Steinkopff, Dresden, 1938), *Chem. Abstr.*, 33, 3402 (1939).

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benzamidomalonate. Wood & du Vigneaud described a new method of preparing *D*-cystine. Johnson synthesized α -amino-pelargonic acid, $\text{CH}_3 \cdot (\text{CH}_2)_6 \cdot \text{CHNH}_2 \cdot \text{COOH}$. Duschinsky reported a synthesis of optically active citrulline, $\text{NH}_2 \cdot \text{CO} \cdot \text{NH} \cdot (\text{CH}_2)_3 \cdot \text{CHNH}_2 \cdot \text{COOH}$. Octopine, a secondary amino dicarboxylic acid containing alanine and arginine residues, was first obtained from animal sources; it has now been synthesized by Knoop & Martius and by Irvin & Wilson, who have also determined its four ionization constants. Shemin & Herbst described a synthesis of *DL*- β -cyclohexylalanine, which is hexahydro-phenylalanine.

Greenstein (2) synthesized diglycyl-*L*-cystine; Greenstein & Klemperer synthesized α -aspartyl-histidine and obtained titration curves and pK' values for both the α - and β -forms. Another peptide, dicholylcystine, and the related cholylcysteic acid were synthesized by Velick, White & Lewis. The isolation of glutathione from yeast was simplified by Schroeder, Collier & Woodward. Peptides occurring in marine algae were studied by Haas, Hill & Russell-Wells.

The pantothenic acid of Williams and co-workers was shown to contain one carboxyl group, two hydroxyl groups, and probably one substituted amide group; its calcium salt was assigned the formula $(\text{C}_8\text{H}_{14}\text{O}_5\text{N})_2\text{Ca}$. Pantothenic acid was also shown (Weinstock *et al.*) to yield β -alanine on cleavage; the amount, as estimated by biological tests, was ninety-one per cent of that required by the preceding formula, and it was concluded that the pantothenic acid was ninety-one per cent pure.

Derivatives and related compounds.—The nickel salts of a number of amino acids were prepared by Lang, who measured their solubilities in water and in methanol at 24.5° ; he concluded that the separation of amino acids as nickel salts was not practical. The combination of cysteine with sugars (pentoses, hexoses, and lactose) was studied by Schubert, who found that compounds were formed in neutral aqueous solution, with the elimination of a mol of water and the production of an acid reaction; the compounds did not respond to the nitroprusside test for thiol groups in sodium bicarbonate solution, but they all could absorb iodine with the production of cystine. Compounds related to canaline $[\text{NH}_2 \cdot \text{O} \cdot (\text{CH}_2)_2 \cdot \text{CHNH}_2 \cdot \text{COOH}]$ and canavanine $[\text{NH}_2 \cdot \text{C}(\text{:NH}) \cdot \text{NH} \cdot \text{O} \cdot (\text{CH}_2)_2 \cdot \text{CHNH}_2 \cdot \text{COOH}]$ were studied by Borek & Clarke; they prepared several carboxylated ethers of hydroxylamine and hydroxyguanidine, finding pK' values in good agreement with those of canaline and canavanine. Christ-

man & Levene continued their studies of proteinogenic alkyl alcamines; by the reduction of esters of amino acids in the appropriate alcohol they obtained the N-dipropyl derivative of leucinol and N-dimethyl-*dl*-phenylalaninol.

Analysis.—Bergmann & Stein (1, 2) have described in detail their ingenious solubility method for the quantitative determination of amino acids in mixed solutions. They have chosen reagents which precipitate the individual amino acids, but not completely. They find that the solubility product of the ions of the compound is constant in any two experiments in which the only variable is the amount of the reagent added; from the weights of the compound precipitated, and the known amounts of the reagent, the amount of the amino acid may be calculated without a knowledge of the exact value of the solubility product. The error of the method appears to be not more than one to three per cent of the total amount of each amino acid. A semi-micro modification of this method has been described by Ing & Bergmann.

The heavy isotope of nitrogen, N^{15} , was put into amino acids by Schoenheimer and collaborators (Rittenberg, Keston, *et al.*). They found that the normal relative abundance of nitrogen isotopes in amino acids is the same as in air, described a method for determining amounts of N^{15} by a mass spectrometer, synthesized amino acids containing several per cent of their nitrogen as N^{15} , and showed that the nitrogen atoms in such amino acids do not change places with those of other nitrogen compounds in aqueous solution, even at 100° . Their subsequent work deals with metabolism, and will not be discussed here.

Other papers dealing with analytical methods include the following: nitration of phenylalanine [Block & Bolling (2)]; micro-estimation of threonine [Block & Bolling (3)]; arginine in mixtures (Thomas, Ingalls & Luck); amino acid nitrogen in blood and urine (Sahyun & Goodell; tryptophane (Shaw & MacFarlane); tyrosine, tryptophane, and cystine (Bálint); arginine and histidine (Mouroto & Hoffer); ninhydrin method for amino acids (Virtanen & Laine); glutamic acid (Cohen); glutathione (Ennor); decomposition of cystine and cysteine in boiling solutions [Routh (1, 2)]; polarographic method for cystine (Stern, Beach & Macy); colorimetric and polarographic methods for cystine (Sullivan, Hess & Smith); identification of amino acids by means of 3,5-dinitrobenzoyl chloride (Saunders); determination of amino nitrogen using a copper method and a thiosulfate

titration (Pope & Stevens); semimicro Kjeldahl distillation apparatus (Redemann); the function of iodine in amino nitrogen analysis by the nitrous acid method (Dunn & Porush); instability of cystine toward alkali (Jones & Gersdorff).

Physical chemistry.—The crystal structure of glycine was studied by Albrecht & Corey, using the x-ray method; they found evidence for a zwitterion structure in the crystals. A form of glycine believed to be truly undissociated and not zwitterionic was obtained by Przylecki and co-workers, who precipitated it by ether from solution in glacial acetic acid; it was an amorphous powder, of a lower density than the usual form, and x-ray analysis indicated a larger elementary cell. The infrared absorption spectra of amino acids, in powder layers, were studied by Wright; he concluded that the *dl*-form of an amino acid is a compound and not a mixture of the *d*- and *l*-forms, and that interaction between carboxyl and amino groups was indicated.

A study of the interaction between glycine or alanine and chlorides of the alkaline-earth metals, in aqueous solution [Joseph (3)], indicates that reliable activity coefficients for such salts may be obtained from a cell without liquid junction, including a silver chloride electrode and an electrode of the third class: lead amalgam, lead oxalate, alkaline-earth oxalate. The lead oxalate is applied in the form of a paste, held between two cellophane membranes which separate the amalgam and the solution. It seems possible that such a cell may yield useful information about the state of calcium in physiological fluids.

Dielectric constants of amino acid solutions were measured by Fricke & Parts. Greenstein & Wyman found a correlation between dielectric increments, apparent molal volumes, and pK' values of aliphatic amino acids; similar studies with cystine peptides (diglycylcystine) were made by Greenstein, Klemperer & Wyman. Wyman (3) has been able to calculate the dielectric increment of lysylglutamic acid, a peptide containing two positive and two negative charges, on the assumption of free rotation about the single bonds of its chain. The effect of pH on the dielectric constants of amino acids was investigated by Carr & Shutt, who attempted to calculate pK' values from their curves; the results seem to indicate that this is not a good method for the determination of ionization constants.

A mathematical calculation by Westheimer & Shookhoff, of the ratio of the ionization constant of an amino acid to that of the cation of a salt of its ester, indicates that their theory accounts fairly satis-

factorily for the pK' values of other workers. Electrometric titration curves and pK' values were obtained for hydantoin and ten related compounds by Pickett & McLean, who concluded that in most cases only the N-3 hydrogen atom was removed by dissociation.

Kirkwood has extended the theory of dipolar ions to include the limiting laws applicable to dipolar ions of elongated shape. His discussion is limited to aliphatic amino acids and peptides. An experimental comparison of a dipolar substance, glycine, with its uncharged isomer, glycolamide, was made by Gucker, Ford & Moser. They measured specific heats and densities of aqueous solutions, finding that the uncharged isomer has higher values for both the apparent molal heat capacity and the apparent molal volume.

Interactions between dipolar ions in aqueous solution were studied by Cohn, McMeekin, Ferry & Blanchard. They measured the solubilities of asparagine and cystine in solutions containing varied amounts of a more soluble amino acid or other dipolar substance. The effect of a soluble amino acid on its own activity coefficient was compared with its effect on that of the slightly soluble dipole. In each case a straight line was obtained when the molar decrease in the logarithm of the activity coefficient was plotted against the ratio, D_0/D , of the dielectric constants of the solutions. Such a linear relation was expected from theoretical considerations, but it was not possible to calculate the constants of the various linear equations from the present theory of dipolar ions.

The ionization constants of amino acids, the dielectric constants of their solutions, and the electrophoresis of proteins were discussed by MacInnes in his book on the principles of electrochemistry. Harned & Owen reviewed the results obtained by their method for the determination of the thermodynamic dissociation constants of weak electrolytes by means of cells without liquid junctions; their tables include values obtained for nine amino acids over a wide temperature range (Owen; Nims & Smith; Smith, Taylor & Smith). The amphoteric properties of amino acids and proteins were discussed by Hitchcock (also in Schmidt, Chapter XI).

PROTEINS

Purification, fractionation, and isolation.—Electrodialysis, with mercury electrodes separated from the solution by cellophane membranes, was used by Joseph (2) for the purification of proteins or amino acids. A simple cell for the separation of albumin and globulin

by cataphoresis was described by Coolidge. Automatic apparatus for drying large amounts of proteins from the frozen state was designed by Greaves & Adair.

A crystalline albumin of constant solubility was obtained by McMeekin from carbohydrate-free crystals of horse serum albumin; this initial material gave two boundaries in electrophoresis at pH 4, as did the corresponding fraction of human serum albumin (Luetscher). Improvements in the sodium sulfate method for the estimation of serum albumin and globulin were described by Robinson, Price & Hogden (1, 2); photoelectric measurements of turbidity were also used for this purpose (Looney & Walsh). Sodium sulfite was found to give about the same fractionation of horse serum as other salts (Roche, Derrien & Moute). The serum proteins of the goat (Reinecke, Peterson & Turner) and of certain reptiles and fishes (Roche, Derrien & Chouaïech) were also fractionated. Kleczkowski obtained four albumin fractions of horse serum, of which only one was free from lipoid and carbohydrate; he affirmed the existence of lipoproteins. Hewitt studied the polysaccharide of serum proteins; it seems to contain galactose-mannose-acetylhexosamine.

Two crystalline myogens, A and B, were obtained from rabbit muscle by Baranowski (1, 2); his myogen-A was ultracentrifuged by Grålen, who found it to be monodisperse, with a molecular weight of 136,000 to 150,000.

The proteins of electrical tissue (*Torpedo*, etc.) were studied by Bailey, who found them to differ from muscle proteins; myosin was absent, and they were associated with much lipoid.

Additional papers include: copper-proteins from blood and liver (Mann & Keilin); flavoproteins from milk (Corran & Green) and from heart muscle (Straub); calf thymus nucleohistone (Carter & Hall); preparation of fibrinogen (Ferguson & Erickson); fractionation of casein [Cherbuliez & Jeannerat (1, 2)]; action of sodium hydroxide on caseinogen (Plimmer & Lawton); fractionation of gluten (Spencer & McCalla).

The purity of crystalline pepsin preparations was questioned by Steinhardt, who found decreasing solubilities on successive extractions with acid salt solutions. He and others found evidence for non-protein nitrogen and inactive protein impurities in the crystals. Northrop stated in his book (p. 160) that repeated precipitation of the crystals yielded a preparation which had a constant solubility and was strictly homogeneous in the Tiselius electrophoresis apparatus. Kunitz

& Northrop pointed out that Steinhardt's experiments were carried out at pH 2.7, where the non-protein component was insoluble, while Northrop's experiments were carried out at pH 4.6, where the non-protein impurity was soluble and could be removed. Hofmann & Bergmann (1) reported the isolation, from beef pancreas, of a crystalline protein which they called heterotrypsin, because its activity toward certain synthetic substrates appeared to be different from that of trypsin. These workers (2) later withdrew this claim, since they found that the very rapid action of trypsin on one substrate had caused them to believe that no action had occurred. Other enzyme papers include the following: crystalline catalase from beef liver (Sumner & Dounce) and from horse liver (Dounce & Frampton); crystalline papain from papaya latex (Balls & Lineweaver); crystalline ribonuclease protein from beef pancreas (Kunitz); crystalline copper-protein, with some tyrosinase activity, from the wild mushroom (Dalton & Nelson); crystalline protein fraction of the carbohydrate-oxidizing enzyme of fermentation (Warburg & Christian); purification of bacteriophage (Kalmanson & Bronfenbrenner).

Composition.—The amino acid composition of secretin, as recalculated by Niemann, seemed to be in accord with Bergmann's periodicity hypothesis. Block & Bolling (1) found a constant ratio for the lysine and arginine content of pseudokeratins (gorgonin, spongin, turtle scutes). Block reported relatively constant mol ratios of histidine, arginine, and lysine in keratins (hair, wool, horn). Human enamel protein (from teeth) was found by Pincus to differ from characteristic keratins in its low sulfur content. The amounts of seven amino acids in erythrocyte posthemolytic residue (stroma) were found to be practically identical for five mammalian species (Beach, Erickson, Bernstein, Williams & Macy); the blood globins of the same species, however, showed specific differences in total sulfur, cystine, and methionine (Beach, Bernstein, Hummel, Williams & Macy). In rabbit myosin, amino acids were found which accounted for about 76 per cent of the nitrogen (Sharp). Hess & Sullivan found that deamination did not change the cystine content of proteins; in this case Sullivan's method gave discordant results, since it depends on free amino and carboxyl groups as well as sulphydryl. Sullivan & Hess found twelve per cent of cystine in insulin if they used formic acid with hydrochloric acid in the hydrolysis, to prevent loss of sulfur. Theorell (1) isolated *l*-cystine from porphyrin-c and (2) obtained artificial complexes of porphyrin and *l*-cysteine; he con-

cluded that the hemin and porphyrin in cytochrome-c were not joined by nitrogen or sulfur. Fischer & Hultzsck found evidence of loosely bound ("masked") iron in phosphoproteins and nucleic acid; it differed from inorganic dissociable iron and from organically bound iron (as in hemoglobin). From the ultraviolet absorption of sheep thyroglobulin, Ginsel concluded that this protein contains diiodotyrosine and thyroxine in the ratio of 2:1. The ultraviolet absorption spectrum of papain (Fruton & Lavin) did not show the bands of tyrosine, although tyrosine was found after hydrolysis of the protein.

Kögl & Erxleben reported that considerable fractions of the amino acids of tumor proteins, especially glutamic acid, occur in the "unnatural" *d*-form. This was not confirmed by Chibnall and co-workers, who found glutamic acid from such tissues to have only the normal (+) rotation of the *l*-form, but Arnow & Opsahl found some *d* (—). White & White pointed out that Chibnall had used a method of extraction differing from that of Kögl, and stated that they had confirmed the observations reported from both laboratories. Chargaff found only the normal configuration for glutamic and aspartic acids from pathogenic bacteria.

Compounds or derivatives.—Biologically active thyroxine was obtained from casein and other proteins by treatment with iodine and hydrolysis (Ludwig & Mutzenbecher; Harington & Rivers). The combination of hemoglobin with the ions of copper, zinc, and mercury was studied by Rawlinson (1, 2). Compounds of casein with gold and cobalt were reported by Jesserer & Lieben. Michael studied the precipitation of proteins by complex cations or anions, especially those containing cobalt and chromium; he found evidence of stoichiometric combination, in more or less the same proportions as the combination with acid or base. Complex precipitates of proteins with added maltose were obtained by Przylecki & Cichocka, who found the maltose content of the complexes to be approximately parallel to the lysine content of the proteins; no complexes were obtained with sucrose. Matula prepared methylated gelatin, its carboxyl groups being presumably esterified by treatment with diazomethane; its acid-binding capacity was unchanged by this treatment, but the determination of alkali-binding, which might have been zero, was complicated by hydrolysis. Sandor & Goldie acetylated serum proteins and diphtheria antitoxin with ketene; they concluded that the primary amino groups could be completely acetylated without changing the phenolic groups, and that progressive acetylation caused a progressive decrease in the

response in immunological tests. Talmud found that egg albumin could bind glycine ethyl ester, and concluded that the protein molecule held 52 molecules of the ester on its outer surface and 150 on the inside; this result was said to be in accord with the cyclol hypothesis of Wrinch. Felix & Mager inactivated pepsin by means of trypsin or acid, dialyzed, and from the concentrated dialyzate obtained, on the addition of clupein, a precipitate having peptic activity; they inferred that pepsin probably contains a non-protein prosthetic group.

Chemical reactions and colloidal behavior.—The reactions of a protein with acid or base were studied in a new way by Theorell & Åkesson; they found that purified cytochrome-c, brought to various pH values, gave a curve of light absorption against pH resembling a stepwise titration curve, indicating four states of ionization and three pK' values. The conductivity of gelatin in hydrochloric acid solutions was measured by Lewis & Broughton; their data indicated that the ionic conductivity of gelatin passed through a maximum value of 15.9 at pH 3.3. The heat of reaction of wool with chloroacetic acid was found by Speakman & Stott to be greater than that produced with hydrochloric acid. The base-binding capacity of wool was studied by Harris & Rutherford. The interaction of proteins with calcium chloride was studied by Joseph (1), who used a calcium amalgam electrode separated from the protein solution by a cellophane membrane. Wyman (1) made acid-base titrations of oxyhemoglobin at three temperatures, and calculated the apparent heats of dissociation; a plot of the latter against pH gave a curve which rose in three steps. From his analysis of the results he concluded that carboxyl groups were titrated below pH 5.5, imidazole groups of histidine from pH 5.5 to 8.5, and either amino or guanidino groups above pH 8.5.

By the use of water with more or less than the usual amount of the heavy isotope of oxygen, O^{18} , Mears & Sobotka found that amino acids and crystalline pepsin could exchange only the oxygen of their carboxyl groups in acid solutions, but that egg albumin did not exchange its oxygen. Insulin in liquid ammonia was found by Roberts to retain its biological activity for one day, but if there was a slight reduction by sodium the activity was lost.

The reactions of silk fibroin with acid and basic dyes were studied by Hoffman & Mack, who found that Freundlich's adsorption equation was followed. The adsorption of salts from solution, on gelatin previously dried at 110° , was found by Docking & Heymann to fol-

low a lyotropic series. The electrical conductance of salts in aqueous gelatin was found to show no abrupt change as the sol set to a gel (Taft & Malm); it was inferred that the gels had a fibrillar structure. Gel structure was also studied (Friedman & Shearer) by measuring the effect of non-electrolytes on the time of setting of gelatin; it was concluded that those gels which set more slowly had a more open structure. The effect of warm water on collagen fibers was interpreted by Cherbuliez, Jeannerat & Meyer as due to two processes, a reversible collapse of a lattice arrangement and an irreversible partial hydrolysis. The existence of bound water in protein systems was reaffirmed by Nicloux, largely on the basis of experiments on the distribution of alcohol between aquatic animals and the external medium.

Ultrafiltration of protein solutions containing salts was studied by Bigwood & Errera, who concluded that the distribution of ions tended to follow a Donnan equilibrium and could be explained without using the hypothesis of non-dissolving space in protein solutions. Egg albumin containing potassium chloride or iodide was ultrafiltered by Trautmann, who found decreasing halide concentrations in successive fractions of the filtrate and assumed electrostatic repulsion of these negative ions by the negative albumin ions.

Denaturation.—The effects of temperature on the denaturation of proteins and the inactivation of enzymes were discussed in terms of the modern theory of absolute reaction rates (Eyring & Stearn). The denaturation of edestin, excelsin or globin by guanidine hydrochloride, urea or their derivatives was shown to be accompanied by the liberation of sulfhydryl groups [Greenstein (1)]. In the case of egg albumin, various salts of guanidine caused the appearance of sulfhydryl groups to different degrees, and in some cases their denaturing effect was augmented by potassium halides [Greenstein (3)]. The denaturation of myosin by similar reagents was accompanied also by the disappearance of double refraction of flow and a decrease in viscosity, but not by a change in solubility (Edsall, Greenstein & Mehl). Alkyl sodium sulfates (C_{10} to C_{18}), and also bile salts, were found by Anson (1) to denature egg albumin and hemoglobin at their isoelectric points without causing precipitation. Anson (2) found under certain conditions a definite reaction between denatured egg albumin and potassium ferricyanide in the presence of sodium dodecyl sulfate; there was evidence that this reaction was due to cysteine sulfhydryl groups in the denatured protein. The denaturation of egg albumin and edestin was found by Hendrix & Dennis to be accompanied by

a decrease in nitrogen content, without the removal of nitrogen; they suggested that the change was due to the addition of water during denaturation. Roche & Donnat found that the denaturation of hemoglobins by sodium salicylate was reversible, while coagulation by trichloroacetic acid was not. The denaturation of serum albumin by urea was studied (Neurath & Saum) by measurements of diffusion and viscosity; dimensions of an ellipsoidal molecule, $20 \times 359 \text{ \AA}$, were calculated.

Diffusion.—The calculation of molecular weight from diffusion measurements by the Stokes-Einstein equation was shown (Friedman & Carpenter) to lead to the correct result for glucose if the diffusion coefficients were extrapolated to infinite dilution by a linear plot against the square root of the concentration. The effect of pH on the rate of diffusion of gelatin (Friedman & Klemm) could be abolished, between pH 2 and 6, by the presence of potassium chloride, which gave values identical with those obtained at the isoelectric point without salt. Diffusion constants for 32 proteins were obtained by Polson (1), who used the method developed in the Upsala laboratory; he found little effect of protein concentration in the case of proteins of high molecular weights (millions), and by combining the diffusion data with Svedberg's sedimentation constants he calculated specific volumes and molecular weights of the protein particles. Neurath made a calculation, using Svedberg's molecular weights and diffusion constants, of the dimensions of protein molecules, assumed to be ellipsoidal; the minor axes were 16 to 64 \AA in length, the major axes varied from 43 to 950 \AA for different proteins, and the ratio of axis lengths varied from one to twenty. Diffusion measurements of tobacco mosaic virus protein in urea solutions led Frampton & Saum to infer a molecular weight of the order of 10^5 instead of 10^7 or more.

Viscosity.—The familiar equation of Einstein for the viscosity of solutions or suspensions containing spherical particles was modified by W. Kuhn in 1932 for the case of rod-shaped particles. Kuhn's equation is

$$\eta = \eta_0 \left[1 + 2.5 G + \frac{G}{16} \left(\frac{s}{d} \right)^2 \right],$$

where η/η_0 is the relative viscosity, G is the fraction of the total volume occupied by the particles, and s/d is the ratio of the length to the thickness of the rods. Kuhn stated that his equation was not exact, and that the factor $1/16$ might be too small; for a net-like

structure of crossed rods, he replaced it by $1/8$. This equation was modified empirically by Polson (2) for the case of protein solutions in such a way as to make it give, in combination with diffusion data, molecular weights agreeing with those obtained by Svedberg's ultracentrifugal methods. Another empirical modification of the Einstein equation was found by Fahey & Green to fit their viscosity measurements of the protein fractions of horse serum. They found that the viscosities of five protein fractions differed so greatly as to be characteristic, and stated that the application of Kuhn's equation yielded ratios of the length to the width of the molecule which varied from about eight for serum albumin to twenty for the most viscous globulin. Frampton (1) concluded that Kuhn's equation could not be applied to the tobacco mosaic virus protein because the particles were not dynamically independent and the apparent viscosity varied greatly with the pressure applied in the measurement. Frampton (2) later pointed out that his data gave an extrapolated value of zero for the ratio of particle dimensions. This value, combined with sedimentation data, led to an infinite molecular weight for this protein, while diffusion data, on similar treatment, gave a corrected molecular weight of zero. The tobacco mosaic protein was also studied by Robinson, who used a Couette rotating cylinder viscosimeter; he obtained a new theoretical equation which gave 87 for the ratio of molecular dimensions instead of the figure of 35 advocated by Lauffer & Stanley on the basis of Kuhn's equation. The elongated shapes of virus proteins, together with their dipole moments, were stated to be responsible for the fact that their solutions exhibited the Kerr electro-optical effect: double refraction was produced by an alternating electric field (Lauffer). Virus proteins have been reviewed elsewhere by Lauffer & Stanley and by Wyckoff, as well as in this volume (p. 545) by Stanley.

Molecular weights.—The average particle weight of urease, as given by osmotic pressure measurements, appeared to be 700,000, while diffusion measurements indicated that particles with a weight as low as 17,000 possessed urease activity (Hand). Apparatus for the measurement of the osmotic pressure of proteins in small volumes of solution was described by Hepp and by Bourdillon. Hepp's apparatus was modified and tested by Peters & Saslow, who found it to yield in two hours results similar to those previously obtained in experiments lasting for days or weeks. If this method yields equally good results in other laboratories, it should provide valuable infor-

mation as to the average molecular weights of proteins. In the interpretation of osmotic pressures of proteins in solutions containing electrolytes, it may be necessary to consider the "buffering effect" observed by Kern, who worked with polyacrylic acids containing fifty to two thousand carboxyl groups as models of proteins. He found that these materials, partly neutralized by sodium hydroxide, yielded osmotic pressures or freezing point depressions as low as 0.2 of that calculated from the concentration of sodium ions alone. When the acid groups were more than 0.3 neutralized, there was relatively little effect of additional alkali on the osmotic pressure; this peculiar "buffering effect" seems not to have been explained.

The measurement of molecular weights by means of the ultracentrifuge was reviewed by Mutzenbecher. Simplified opaque and transparent ultracentrifuges were described by McBain & Leyda, McBain, and McBain & Lewis. The Svedberg ultracentrifuge was applied by Brohult & Claesson in a study of a hemocyanin of molecular weight 6,700,000; they found that the dissociation of this protein into particles of $1/2$, $1/8$, and $1/16$ of this size could be brought about, with complete reversibility in some cases, by the addition of salts as well as by nonelectrolytes or by changes in pH. Reversible dissociation was also observed by Lundgren, and by Lundgren & Williams, on lowering the salt content of thyroglobulin solutions by dialysis; they used the Svedberg apparatus. Lundgren, Pappenheimer & Williams reported that the diphtheria toxin protein was nearly homogeneous in the ultracentrifuge and in electrophoresis, its molecular weight being 72,000. Abraham found that egg-white lysozyme was homogeneous in the ultracentrifuge but not in solubility experiments; he gave its molecular weight as 18,000. The specific anti-sheep hemolysin of rabbit serum was found by Paic to have a sedimentation constant, determined in the ultracentrifuge, which would correspond to a molecular weight of 420,000 if the particles were spherical. An extensive table of the molecular weights of proteins was given by Svedberg in 1938. In that table the ultracentrifugal molecular weights were compared with calculated multiples of 35,200, although egg albumin, which seems to have furnished the original basis for the multiple unit theory, was assigned a value of 40,500 or 43,800 on the basis of revised sedimentation and diffusion data. Even more striking was the upward revision of the molecular weights of the seed globulins (edestin, excelsin, amandin) from about 200,000 to 300,000; the lower values for excelsin and amandin appeared to be confirmed by the osmotic

pressure measurements of Burk. The molecular weights of hemoglobin and serum albumin, on the other hand, seem to remain fixed at about twice the original Svedberg unit, in agreement with the figures originally obtained by Adair from his osmotic pressure measurements.

Electrophoresis.—The ingenious moving boundary apparatus of Tiselius (mentioned altogether too briefly by that author in the preceding volume of this *Review*) has already yielded valuable results in various laboratories in the study of proteins. Modified methods of obtaining photographic records of electrophoresis in the Tiselius apparatus were described by Svensson and by Longworth, and Longworth & MacInnes gave a clear account of the principles underlying the method and the experimental procedure involved in its use. Tiselius & Horsfall used the apparatus to study dissociation and reassociation in hemocyanin solutions, and found evidence for the production of mixed molecules by combination of fragments of hemocyanins from different species. Stenhagen & Teorell found that thymonucleic acid, with a molecular weight of about 200,000, had an ionic mobility nearly equal to that of the acetate ion; when mixtures of thymonucleic acid and serum albumin were electrolyzed, it was found that a yellow pigment, not separable from the albumin by electrophoresis of the latter alone, now left the more slowly moving protein. Kekwick found that in the Tiselius apparatus the proteins of normal human serum appeared to consist of 59 per cent of albumin and 41 per cent of globulins (α , β and γ), while in the ultracentrifuge the corresponding figures were 78 and 22; the difference was not explained. By the electrophoresis of antipneumococcus horse serum, Moore, van der Scheer & Wyckoff found the antibody activity to be associated with the γ -globulin. Rat serum in the Tiselius apparatus, according to Jameson & Alvarez-Tostado, showed only two globulin fractions instead of the three found with human or horse serum. The apparatus was applied by Mellander to the study of milk proteins; four boundaries were observed, and casein fractions of different phosphorus content were obtained. The Tiselius method was also used by Shipley, Stern & White to follow the separation of anterior pituitary proteins; crystalline prolactin was shown to be homogeneous in migration, but to gain a second component on storage.

The older method of electrophoresis in a U-tube at 25°, with visual observation, was used by Davis & Cohn in a study of carboxyhemoglobin; they found that variations in the ionic strength (0.02 to 0.20) of the phosphate and citrate buffers used had about as much influence

on the mobility as did variations in pH (5.65 to 7.2). The apparent isoelectric points varied from below pH 6.0 nearly to pH 7.0, with the curves pointing toward considerably higher values at zero ionic strength. Their results, which were checked to some extent with the new Tiselius apparatus, serve to emphasize the idea that the isoelectric point of a protein is not necessarily a constant if more than one variable enters the experiments.

Abramson, Gorin & Moyer have again compared their mobilities of protein-coated particles with those obtained by Tiselius for proteins in solution; their paper includes some new calculations of ionic radii and length to breadth ratios for three proteins. Microscopic observation of suspended particles has also been applied by Highberger and by Beck & Sookne to determine the isoelectric point of collagen, and by Sookne & Harris (1, 2) to determine the isoelectric points of silk proteins and of wool.

Dielectric properties.—Fricke & Jacobsen measured dielectric constants and dielectric absorption in gelatin-water systems; they concluded that the dielectric properties depend on polarization at interphases, originating in oriented water molecules. Elliott & Williams were able to account for the dielectric constants of aqueous-alcoholic solutions of zein by assuming for this protein a molecular weight of 38,000 and an ellipsoidal molecule with axis lengths in the ratio of 1:7. Arrhenius studied the effects of glucose, glycerine, urea, and glycine on the dielectric behavior of carboxyhemoglobin solutions, and found that the added substances lowered the dipole moment slightly. Cohn, Ferry, Livingood & Blanchard made a preliminary report on the solubility and dielectric properties of insulin and its crystallization with radioactive zinc. The review by Cohn (3), on physicochemical characteristics of protein molecules, includes a discussion of dielectric constant dispersion methods and the size and shape of the molecule.

Hemoglobin and related proteins.—Wyman (2) studied the effect of temperature on the acid-base titration of both oxygenated and reduced hemoglobin, and calculated the heat of oxygenation; he was able to account for his results on the basis of a dissociation of hydrogen ion accompanying oxygenation, the heat of dissociation having a value characteristic of the imidazole group of histidine. Ross (1, 2) found that the course of digestion of carboxyhemoglobin by pancreatic enzymes was different from that of oxyhemoglobin, and that the former yielded a product of molecular weight 1060 or 2120, apparently containing the original heme-protein linkage of hemoglobin. Hemo-

globin solutions of high oxygen capacity were obtained by Altschul, Sidwell & Hogness after the removal of impurities with γ -aluminum hydroxide. Altschul & Hogness determined the oxygen saturation curves of such material, which was not crystallized, and discussed the results in terms of mass law constants and interaction between heme groups; they did not consider Hill's equation to be applicable. Hall described a spectroscopic method, with a lamp bulb inside of a double-walled tonometer, for determining the oxygen saturation of whole blood. Hissey & Morrison studied the oxygen uptake of hemoglobin which had been dried by vacuum distillation at 38° ; it could take up oxygen or carbon monoxide if not exposed too long to the air. Perutz investigated the absorption spectra of single crystals of hemoglobin in polarized light, and concluded that methemoglobin and oxyhemoglobin have four parallel heme groups. Coryell, Pauling & Dodson made magnetic measurements during the titration of oxyhemoglobin to ferrohemoglobin, and of the latter (reduced hemoglobin) to the nitric oxide compound; they found no evidence of chemical interactions between the ferroheme groups. Coryell studied the existence of chemical interactions between the hemes (hemins) in ferrihemoglobin (methemoglobin) and the role of interactions in the interpretation of ferro-ferrihemoglobin electrode potential measurements. Taylor measured the magnetic susceptibility of myoglobin and ferrimyoglobin, and found the same magnetic moments, per atom of heme iron, as in the corresponding hemoglobins. Because myoglobin contains only one or two hemes per molecule in place of the four in hemoglobin, he concluded that there was no magnetic interaction between the hemes in hemoglobin. Sulfhemoglobin was prepared by Michel from hemoglobin by treatment with a soluble inorganic sulfide and hydrogen peroxide; a similar sulfur derivative could be prepared from muscle hemoglobin.

Effects of radiation.—Svedberg & Brohult found that hemocyanin could be split into halves by α -particles, with one molecule split by each α -particle; the rate was the same at room temperature and at the temperature of liquid air. At room temperature hemoglobin and serum albumin were altered by secondary reactions and yielded non-homogeneous solutions. At the lower temperature these proteins were practically unchanged. Bernhart (1) found that ultraviolet light had little or no effect on the pH of acid solutions of egg albumin, but that it caused a marked decrease in the pH of alkaline solutions of this protein. This decrease in pH was accompanied by nearly com-

plete denaturation, and it seemed to require the presence of oxygen; irradiation under nitrogen produced a change in the opposite direction. Bernhart (2) also followed the kinetics of this denaturation, and found it necessary to assume that two first order processes were involved. The kinetics of the destruction of tyrosine in egg albumin by ultraviolet light (Bernhart & Arnow) could be fitted by assuming first order destruction and zero order formation of a phenolic substance. Ultraviolet light, but not soft x-rays, caused changes in the light absorption and sedimentation in the ultracentrifuge of human serum albumin (Sanigar, Krejci & Kraemer).

Surface films.—A review of the literature on surface films of proteins, formed spontaneously from solutions or by a spreading technique, was published in 1938 by Neurath & Bull. The effect of ultraviolet light on protein monolayers was studied by Mitchell & Rideal, who reported increases in the phase-boundary potentials, in liquefaction, and in the surface pressure at constant area. Schaefer described characteristic expansion patterns of individual proteins spread in monolayers on water partly covered by oil films. Langmuir & Schaefer reviewed their work on protein monolayers, and Langmuir (1) gave an elementary lecture on protein films and a more extensive lecture (2) on molecular layers. Rideal delivered an address on film reactions as a new approach to biology. Certain precautions needed in determining the thickness of protein monolayers by the multilayer dipping technique were pointed out by Dean, Gatty & Stenhagen. The surface viscosity and elasticity of unimolecular films of casein and of a nerve protein were measured by Fournet. Protein films at a benzene-water interface were studied by Alexander & Teorell, who measured interfacial tension by a ring method and interfacial potential differences by an ionization method; they found that protein films between these two liquids were more expanded than at an air-water interface, and they were able to obtain complete force-area curves in twenty minutes by successive injections of protein at the interface. Bateman & Chambers studied the surface elasticity of films of egg albumin, which they defined as

$$M_s = -A \frac{dF}{dA}.$$

It was suggested that (M_s, F) curves furnished a better characteristic description of protein films than the usual (F, A) curves (A is surface area, F is surface pressure or force per unit length). They con-

cluded that the surface elasticity of egg albumin on liquids of pH 0.65 to 11.7 is due to a unimolecular film of true limiting area 1.0 sq. m. per mg. Dognon & Piffault reported the absence of denaturation by heat of serum albumin on the surface of a solution; the surface tension of the film showed no sudden change as the temperature was raised to 70°.

Structure.—The structure of proteins, as inferred from x-ray studies, was reviewed by Krüger and by Astbury (1, 2). It seems to be generally agreed that fibrous proteins such as keratins consist principally of polypeptide chains, either extended or folded. For denatured proteins or protein films the polypeptide chain structure is also favored. These chains are presumably held together laterally by some sort of forces, and Astbury has suggested that they may fold into closed hexagons even in certain types of fibrous proteins. The thickness of a monolayer of egg albumin appears to be about 9.5 Å (Astbury), while the dimensions of the egg albumin molecule in solution are of the order of 44 Å (Svedberg). Undenatured proteins in solution are called globular or corpuscular; this does not mean that the molecules are spherical, for many globular proteins are believed to be ellipsoidal or even rod-shaped. The dimensions of these particles are such, however, that it has been difficult to picture them as made up simply of thin polypeptide chains, without some means of holding the chains in a three-dimensional form.

One picture of the structure of globular proteins is given by the cyclol hypothesis of Wrinch [Langmuir (3), Langmuir & Wrinch], according to which the polypeptide chains are replaced by a fabric or cage of hexagons formed by a sort of enolization of carbonyl and imino groups from different peptide linkages. Langmuir (3) claimed that this theory was supported by x-ray measurements of insulin crystals, but his interpretation of these diagrams was questioned by Bernal (1, 2, 3). Bragg also criticized the conclusions of Langmuir & Wrinch concerning the structure of insulin; he pointed out that their solution of the diagrams depended on assumptions which had not been justified. Pauling & Niemann made calculations of bond energies which convinced them that the cyclol structure was unstable, and concluded that proteins did not have such a structure. Numerous other arguments on this subject appeared during the year, largely in the pages of *Nature*.

An x-ray study of collagens from different animal sources was made by Champetier & Fauré-Fremiet (1); in some cases they found

that collagen precipitated from solution in acicular form gave the same spacing as fibers of the natural substance. The same authors (2) concluded that secreted keratins from various animal forms showed none of the characteristics of α -keratins, but were composed of peptide chains of the collagen type.

Miscellaneous papers.—Rotatory dispersion of gelatin in urea solutions (Carpenter & Lovelace); effect of ultrasonic waves on the optical rotation of gelatin (Sata); dynamic birefringence of serum proteins in glycerol (Sadron, Bonot & Mosimann); disintegration of erythrocytes and denaturation of hemoglobin by high pressures (Dow & Matthews); ultrasonic centrifuge for extracting enzymes, viruses and hormones (Girard & Marinesco); a review on active proteins and peptides (Dirschel); serological specificity of keratins (Pillemer, Ecker & Wells) and of keratine derivatives (Pillemer, Ecker & Martensen); chemical aspects of the precipitin and agglutinin reactions (Heidelberger).

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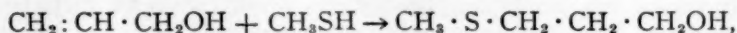
THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF SULFUR¹

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The present review deals primarily with the sulfur of proteins, i.e. that of the sulfur-containing amino acids. Chief among other biologically important sulfur compounds, which are thus omitted, are thiamine and the sulfanilamide class of compounds, both of which, however, are receiving elsewhere in this volume more adequate treatment than could be accorded to them in this chapter. As to the subheadings used, the reader is asked to view them as a system of convenience rather than of consistency.

Synthesis.—Wood & du Vigneaud showed that *d*-cystine is advantageously obtained by way of converting *l*-cystine into *S*-benzylcysteine, racemisation with acetic anhydride, and resolution of the formyl derivatives. The difficulties arising from the presence of a mesomodification in racemised cystine are thus avoided. Two conceivable intermediates in the bio-synthesis of taurocholic acid, dicholylcystine and cholylcysteic acid, were prepared by Velick, White & Lewis. In deriving the latter from the former an aqueous suspension of di(triformylcholyl)cystine dimethyl ester was oxidized with bromine to the sulfonic acid. Morgan & Friedmann succeeded in crystallizing *S*-cysteinisuccinic acid, the formation of which by the addition of *l*-cysteine to the double bond of maleic acid had previously been described. Although the reaction involves formation of an additional center of optical asymmetry only one isomer seems to be formed. The same synthetic principle, the possible biological significance of which was pointed out several years ago by Nicolet (1), was utilized by Akabori & Kaneko in the synthesis of methionol, $\text{CH}_3 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2\text{OH}$. This compound was identified as the main aromatic principle of "shoyu," a Japanese soup-flavoring extract, and is believed to originate from methionine through fermentative degradation. The synthetic reaction used,



¹ Literature coming to the writer's attention after October 1939 has been examined in part only.

is slow. However, it was found that oxygen, mercury, and light are catalytic factors, and their application led to a practical procedure (93 per cent yield after one month at room temperature). Yuan & Li synthesized an amino acid derivative of thiophene, β -2-thienyl-

alanine, $\overline{\text{S} \cdot \text{CH} : \text{CH} \cdot \text{CH} : \text{C}} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$, on account of its possible relation to methionine. The same compound, as well as its decarboxylation product, β -2-thienylethylamine, was similarly synthesized by Barger & Easson. The synthesis is based on the condensation of thiophene-2-aldehyde with hippuric acid (Erlenmeyer method). The problem of the biogenesis of thiamin led Harington & Moggridge to consider the possibility of its thiazole moiety originating from methionine, conceivably by the condensation of one molecule each of acetaldehyde, ammonia, and methionine into β -(4-methylthi-

azolyl-5)-alanine, $\overline{\text{S} \cdot \text{CH} : \text{N} \cdot \text{C}(\text{CH}_3) : \text{C}} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$, which by typical fermentative degradation could lead to the thiamin component. They (and simultaneously Buchman & Richardson) therefore synthesized the postulated amino acid, as well as its decarboxylation product β -(4-methylthiazolyl-5)-ethylamine, using an Erlenmeyer condensation. The preparation of crystalline sulfinic acid from cystine disulfide has been reported by Lavine.

Physical chemistry.—Greenstein *et al.* found that the dielectric increments of their synthetic cystine peptides agree with the assumption of free rotation around the disulfide linkage, while an analysis of the dissociation constants revealed the acidifying effect of sulfur on adjacent functional groups. The properties of cystine disulfide had indicated that oxygenation of sulfur calls forth enhanced acidifying influences (Toennies & Lavine), and similar evidence has now been found for the sulfoxide of methionine [Toennies & Kolb (2)].

Reactivity.—According to Fujita & Numata (2, 4, 5) when solutions of cystine or disulfide glutathione which contain mercuric acetate are saturated with hydrogen sulfide, the disulfides are completely reduced to the thiol form in several hours. In the case of cystine only a small amount of reduction takes place when mercuric ion—which is promptly precipitated as sulfide—is omitted. The catalytic mechanism behind this interesting phenomenon, which recalls the early observation of Mauthner on a similar effect produced by cupric ion, is still awaiting investigation.

Lavine has reported on the oxidation, by iodine and iodate respectively, under different conditions, of cysteine, cystine, cystine disulf-

oxide, and cysteine sulfinic acid. Toennies & Callan have shown that cupric ion, which is a powerful catalyst in the hydrogen peroxide oxidation of cysteine, does not influence the action of peroxide on cystine. They also found that the latter oxidation is favored by increasing acidity which influences the peroxide oxidation of cysteine in the opposite sense.

The addition reaction $(p-)O:C_6H_4:O + HSR \rightarrow C_6H_5(OH)_2-$ (SR) (1:4:2), of interest in connection with the inhibitory action of thiol compounds in the autoxidation of hydroquinones, has been described by Snell & Weissberger. Hellström showed that depending on conditions, the interaction of thiol compounds with halogen-substituted acetic acids may take place in different ways: $RSH + XCH_2COOH \rightarrow RS \cdot CH_2COOH + HX$, $2 RSH + XCH_2COOH \rightarrow RSSR + CH_3COOH + HX$, or $2 RSH + 2 XCH_2COOH \rightarrow RSSR + 2 CH_3COOH + X_2$. Schubert has demonstrated the formation of condensation products of cysteine with various sugars which in their properties resemble the thiazolidines formed by condensation of cysteine with simple aldehydes. Routh has initiated studies on the decomposition of cystine and cysteine in aqueous solution. The extent of hydrolytic cleavage of cystine, with formation of cysteine, hydrogen sulfide, acid products, and sulfur, and likewise the extent of changes suffered by cysteine—formation of cystine, hydrogen sulfide, and acid products, including probably sulfinic acid—appear to be the same regardless of whether the substances are boiled in air or in an atmosphere of nitrogen. No sulfate was formed in either case. Schöberl has continued his studies on the action of alkali on disulfides [Schöberl & Hornung, Schöberl & Rambacher (2)]. It was found that the hydrolysis of β -disulfide carboxylic acids (e.g. cystine) does not tend to follow the pattern previously established for α -disulfide carboxylic acids, $RSSR \rightarrow RSH + RSOH$, followed by elimination of hydrogen sulfide from the sulfinic acid with resulting formation of an aldehyde or keto group. Instead, the sulfhydryl component tends to lose hydrogen sulfide, probably by way of double bond formation, in accordance with Nicolet's (2) views. The sulfinic acid group ($-SOH$) is, at least in part, thought to be stabilized by conversion into a hydroxy group through elimination of sulfur. In conformity with these views it could be shown that on increasing the temperature of alkaline treatment of disulfide glutathione the resulting amount of GSH decreases together with increasing formation of hydrogen sulfide, until at 100° no GSH was formed, but hydrogen

sulfide corresponding to 50 per cent of the total sulfur was obtained. The remarkable lability of cystine in protein linkage is again demonstrated by a study of Neglia, Hess & Sullivan on glutenin prepared by an alkaline treatment. Interesting contributions to the problem of alkali lability were made by Jones & Gersdorff. The extent of destruction of cystine at pH 8 to 9, defined by absence of response to the Sullivan test after hydrolysis, was greatly increased by tryptic digestion at the same pH. The labilization was not confined to the protein cystine but added free cystine suffered extensive degradation and even seemed to increase the extent of damage to the protein cystine. Mutual oxidation-reduction effects between disulfide linkages, their cleavage products and the products of tryptic protein digestion are suggested by the authors as being involved in these phenomena. Tryptic digestion at pH 6.8 caused smooth liberation of the theoretical amount of cystine.

The labilizing influence of neighbouring keto groups on the carbon-sulfur linkage, which has been exemplified in regard to β -keto groups by Nicolet (2) and by Waelsch & Borek is further illuminated by work of Behaghel & Ratz on the labilizing effect of α -keto groups, which in many cases leads to the hydrolytic splitting of the carbon-sulfur bond ($\text{—C—S—} \rightarrow \text{—CH} + \text{HOS—}$).

New reactive potentialities of homocysteine thiolactone, the product of acid demethylation of methionine, were demonstrated by an investigation of du Vigneaud, Patterson & Hunt. They showed that reappearance of reactive sulfhydryl in alkaline solutions does not represent a simple hydrolytic opening of the thiolactone ring, but that by a process of mutual ring opening two molecules of the thiolactone combine to form one molecule of the diketopiperazine of homocysteine. This compound is homologous with the diketopiperazine of cysteine of Greenstein (4) but unlike the latter it does not oxidize into a dimeric didisulfide, but into an amorphous polymerized structure, which, however, on hydrolysis yields homocystine.

Analytical methods.—The possibilities of losses and incomplete oxidation in some of the customary procedures are emphasized by a series of studies on methods of sulfur determination in plant materials (Blanck *et al.*; Rippel & Nabel; Stotz; Balks & Wehrmann). The danger of losing volatile sulfur on drying is shown, and nitric acid digestion followed by alkaline fusion is recommended as a dependable and all-embracing oxidation method (Blanck & Sachse). Masters has studied the same problem from the point of view of food

analysis, giving preference to the nitric-perchloric acid oxidation method originally proposed by Kahane, or to a modification of ter Meulen's hydrogenation method. Sulfur determinations, obtained by these methods, on three hundred different foodstuffs are reported by Masters & McCance. Determination of volatile sulfur in protein food products as a criterion of freshness has been proposed by Die-mair *et al.*

Among specific analytical reactions the sulfhydryl and disulfide determination by phospho-18-tungstic acid and sulfite (modified Folin method) has received further study. Schöberl & Rambacher (1) found that the reactions involved in the disulfide determination are sensitive to variations of temperature and recommend that the procedure be carried out at exactly 20°. They also concluded that the reduction of the reagent by sulfite is inhibited by cystine, because they obtained correct values only when the color produced by sulfite alone was not subtracted from the readings obtained on cystine or cysteine in its presence. A further study, by Schöberl & Krumei, covering a variety of compounds, shows that the facility of the sulfhydryl-producing sulfite cleavage differs for different disulfides and is favored by increasing pH. This was also shown by Micheel & Bode. Elsworth & Phillips demonstrated that the bisulfite ion is the reacting form in the disulfide cleavage. As to the chromogenic reduction of the phosphotungstic acid, Schöberl & Krumei conclude that the minimum pH at which the reaction reaches completion depends on the dissociation constant of the sulfhydryl group. They also observed that in a phosphate buffer much less color may be obtained than in an acetate buffer of the same pH. The kinship of phosphate and phosphotungstate, they suggest, may be involved in this phenomenon.² A factor in these studies which apparently has not been discussed is the pH dependence of the stability of the phospho-18-tungstic acid molecule. It has a half-life time of about forty minutes at pH 5, two minutes at pH 8, and less than one minute at pH 11 (Toennies & Kolb, unpublished). Micheel & Bode emphasize that a test by sulfite-phosphotungstic acid obtained on a protein hydrolysate is no proof for the presence of a disulfide group in the original protein, since a sulfhydryl test is elicited by sulfite from such substances as thiazolidine carboxylic acid and homocysteine thiolactone. The need for cautious interpretation is further emphasized by observations of Micheel &

² However, Hammett & Chapman (1) observed similar interference of phosphate in the nitroprusside reaction.

Emde that on acid hydrolysates of proteins as well as of compounds like cystine and disulfide glutathione determination of sulfhydryl groups by the difference in the phosphotungstate colors obtained with and without the presence of mercuric chloride may be falsified because mercuric chloride, besides masking sulfhydryl groups, may set free other groups (tentatively assumed to be sulfenic acid groups) which reduce the color reagent. Evidence on the retarding action of thiourea in the reduction of phosphotungstic acid [Toennies (1)] likewise shows that one must be on guard against interfering substances.

Some instructive investigations concerning the differential behavior of cysteine and glutathione in the nitroprusside test will tend to revive practical interest in this thiol reaction. Mentzer demonstrated that the two compounds produce equal molar color intensities in a saturated magnesium sulfate solution, that when sodium sulfate is used instead the glutathione color is decreased by about 45 per cent while the cysteine color is increased 42 per cent, and that practical results can be obtained by the application of this principle to mixtures. Fujita & Numata (3), working with saturated sodium chloride solution, showed that the initial color is identical for both compounds, but that the glutathione color is much more stable than that caused by cysteine so that the presence or absence of the latter in tissue extracts, etc., can be deduced from the rate of fading of the nitroprusside color.

As regards other methods of thiol determination, Kuhn *et al.* have shown that on a microscale the iodometric oxidation of sulfhydryl to disulfide with 0.004 *N* iodine solution (in acetic acid) and back titration by thiosulfate of the same concentration gives sharp results when carried out in 70 per cent acetic acid. Fujita & Numata (1) report a carefully elaborated method for the iodometric determination of thiol groups in metaphosphoric acid extracts of tissue. The same authors (5) have developed a specific method for cysteine and (after reduction with hydrogen sulfide in the presence of mercuric acetate) cystine which is based on the production of a methylene blue color by reaction with dimethyl-p-phenylene diamine (Fleming) under conditions in which glutathione does not react. Application of the porphyrinden method of Kuhn & Desnuelle for the determination of thiol groups in proteins has been reported by Greenstein (1, 2), and by Balls & Lineweaver. The latter obtained evidence on papain which raises serious doubt as to the specificity of the reagent for thiol groups. Anson (2)

showed that in the presence of "Duponol" (a detergent mixture of higher alkyl sulfonates) a definite amount of ferricyanide is reduced by ovalbumin at pH 6 to 8, probably by the action of thiol groups, but he also discusses the problem of absolute specificity.

A general question which seems to require attention in connection with thiol determinations in tissue extracts and body fluids is the extent to which the reacting groups may be the result of mechanical and chemical actions involved in the methods. Thus Hammett & Chapman (3) call attention to Hammett's demonstration that sulfhydryl groups are liberated by mechanical trauma of living tissue.

Hess & Sullivan (2) showed that the cuprous oxide method of Vickery & White is not strictly specific for cystine and cysteine since dithiodihydroxydipropionic acid, the product of deamination of cystine, entered practically completely into the cuprous precipitate. Stern, Beach & Macy have studied Brdicka's polarographic cysteine-cystine method on protein hydrolysates. They found that the disturbing effect of other amino acids can be eliminated by the use of a calibration curve obtained through addition of known amounts of cystine to the hydrolysate under investigation. With cystine concentrations of $5 \times 10^{-5} M$ the accuracy was still ± 5 per cent.

A method which would seem to promise usefulness in biochemical work is the alkaline hydrogen peroxide oxidation of Kitamura. This author has shown that under his conditions sulfur linked in the ketonic form ($>C=S$) or in forms susceptible to desmotropic ketonization ($=C-SH$ etc.) is quantitatively converted to sulfate while

genuine $-SH$ or $-S-$ sulfur is not thus split off. Among compounds of biological interest ergothioneine and thiolhistidine should respond to Kitamura's treatment.

An interesting approach to the partition of urinary sulfur compounds has been made by Lefèvre & Rangier. After removing inorganic and organic sulfate and separate determination of thiocyanate they separate the alkaline evaporation residue into an alcohol-soluble and an alcohol-insoluble fraction. The latter is assumed to contain peptides with sulfonic acid ($-SO_3H$) groups, while in the former sulfhydryl, disulfide, and methio($-SCH_3$)-peptides are assumed to be present. The same separation, preceded by bromine oxidation, is thought to leave only the methio-peptides in the soluble fraction, while the sulfhydryl and disulfide peptides, now converted into sulfonic acid peptides, are found in the insoluble fraction. Support for

these conclusions is derived from a second procedure, which is applied to the non-dialyzable residue of the urine. The sulfur not precipitated by basic lead acetate is attributed to sulfonic acid peptides, while the lead precipitate presumably contains the sulfhydryl, disulfide, and methio-peptides. This group is again subdivided by repetition of the lead procedure after preliminary bromine oxidation. Only the methio-peptides would now remain to be precipitated. After making certain allowances for taurocarbamic acid and free cystine and methionine essentially identical values for the three groups were obtained by the two procedures. As further confirmations isolation of the barium salt of cysteic acid from the soluble lead salt fraction of the second procedure after acid hydrolysis, and Baernstein methionine determinations are cited. A photoelectric micromethod for the determination of sulfhemoglobin, based on the fact that this compound does not react with cyanide, has been presented by Evelyn & Malloy.

Protein sulfur.—The problems encountered in the determination of cystine and methionine in proteins, especially when carbohydrates are present, have been emphasized by Bailey and Lugg. These difficulties—humin formation at the expense of cystine, which may occur even in hydriodic acid hydrolysates, and volatile iodide in the Baernstein methionine method arising from carbohydrates or tenaciously retained residues of organic solvents—led Lugg, in an investigation of grass proteins, to adopt, in addition to determinations by the Lugg-Folin, Lugg-Sullivan, and Baernstein methods, a method of differential oxidation, which he based on the finding of Blumenthal & Clarke that under certain conditions cystine sulfur is converted to sulfate by nitric acid while methionine sulfur is not. The cystine and methionine values resulting from these data in conjunction with total sulfur and free sulfate figures were generally 10 to 20 per cent higher than those obtained by the Baernstein method. Bailey's work showed that the total sulfur (about 1.2 per cent) of muscle proteins (myogen and myosin) consists of approximately two-thirds of methionine and one-third of cystine, while the sulfur (about 1 per cent) of the grass proteins investigated by Lugg appears to be equally divided between methionine and cystine. Around one-half of 1 per cent of sulfur, present apparently only as cystine, was found by Mazur & Clarke in the protein materials extractable by formic acid from certain marine algae. Since these would seem to be the first recorded instances of ordinary proteins free of methionine, verification of its absence, which was provisionally concluded from the con-

cordance of total sulfur with reduced² alkali-labile (cystine) sulfur, will be awaited with interest. Brand and associates [Kassell & Brand (2); Brand, Kassell & Heidelberger] were able, by means of their carefully elaborated system of analysis, to account fully in terms of cystine, cysteine, and methionine for the organic sulfur of casein, lactalbumin, and thyroglobulin. They discussed the possibility that sulfinic acid groups may be involved in the sulfur, 7 and 17 per cent of the total respectively, not thus accounted for in edestin and papain. Beach *et al.* have investigated the sulfur components of the blood globins and the erythrocyte membrane proteins of different species. Their results, indicating a cystine content of one, two, or three molecules per protein molecule and a methionine content of three or five molecules, suggest that variations in these protein components represent an important aspect of the species differences of hemoglobins. By contrast the proteins of the erythrocyte membranes of different species show a striking similarity not only in regard to cystine (two molecules) and methionine (three to four molecules) content but also in their content of other amino acids. A protein of low cystine and methionine content (molar ratio 1:2, total sulfur 0.16 per cent) is the elastin of ox ligament, which was analyzed by Stein & Miller.

Two substances of protein nature which are particularly interesting on account of their relatively low molecular weight and high sulfur content are egg-white lysozyme and phalloidin. The former, investigated by Abraham, is an enzyme capable of lysing certain bacteria, of a molecular weight of 18,000 and a sulfur content of 3.2 per cent, about one-half of which appears to represent cystine. The latter is a crystalline mushroom toxin, to which the discoverers, Lynen & Wieland, attributed a molecular weight of only 677 on the basis of ebullioscopic determinations. However, the sulfur analysis, reported by Kuhn *et al.* and showing 3.5 per cent methionine and 15.1 per cent cystine, obviously contradicts the low molecular size. Another enzyme which has finally been crystallized is papain (Balls & Lineweaver). It has a molecular weight of 27,000 and contains 1.2 per cent sulfur five-sixths of which have been identified as cystine.

A sulfur protein of special character is sulfhemoglobin, an investigation of which has been reported by Michel. He showed that the compound is formed, under participation of hydrogen peroxide, from hemoglobin and inorganic sulfide ion in the ratio of one sulfur for each iron atom. The reaction does not involve the protein moiety of the hemoglobin, the sulfur of which is immune against oxidation by

bromine water, while the bound sulfide sulfur is readily oxidized to sulfate. The latter does not occupy the place taken in hemoglobin by oxygen or carbon monoxide and it is not present in the form of a reactive sulfhydryl group.

Methionine.—The increasing awareness of the broad distribution of methionine as a protein constituent and its dietary importance have been accompanied by growing attention to the chemistry of this amino acid. Duschinsky & Jeannerat obtained a 68 per cent yield of *l*(—)-methionine after treating the *dl*-compound with the *d*-amino acid oxidase of Krebs and removing the α -keto- γ -methiobutyric acid, formed by oxidation of the *d*-component, as the dinitrophenylhydrazone. The use of mercuric chloride in the isolation of methionine led Toennies & Kolb (2) to study the nature of the interaction involved. They found that the formation of a precipitate—of the composition $(\text{CH}_3 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COO})_2\text{Hg} \cdot (\text{HgCl}_2)_4$ —requires the presence of both mercuric ion and mercuric chloride molecules, and that other amino acids interfere in the precipitation by engaging mercuric chloride through the formation of soluble complexes. A color reaction, with cupric chloride in hydrochloric acid, which among the natural amino acids appears to be specific for methionine, has been reported by Kolb & Toennies. The analytical possibilities have been enriched by Kuhn *et al.*, who have developed a micromodification of Baernstein's method which permits the determination of less than one milligram of methionine. Details for the preparation of *dl*-methionine sulfoxide have been given by Toennies & Kolb (2) and by Micheel & Schmitz (2). The former showed that the compound may be completely reduced to methionine by hydriodic acid, while its oxidizing action on cysteine is slow, and the latter (3) demonstrated its reduction by sulfite. The interesting problems posed by the possibility of four stereoisomers, owing to the asymmetry of the sulfoxide sulfur atom, have not as yet been studied. Toennies & Callan showed that methionine is far superior to cystine and even (in the absence of metal catalysts) to cysteine in its reactivity with hydrogen peroxide. It remains to be seen if this property is of significance from the physiological point of view. The reaction was found to be independent of cupric catalysis, increasing in velocity with increasing acidity, without, however, exceeding the sulfoxide level except in the presence of molybdate ion. Nicolet & Shinn showed that methionine shares with cystine and the hydroxyamino acids the susceptibility to oxidation by periodic acid.

Even though *in vitro* the tendency for the methio group of methionine to cleave in such a way as to retain the sulfur with the carbon skeleton seems to prevail it is very probable that in metabolism the alternative cleavage, with formation of methyl mercaptan, also takes place. A possible pathway for this process was shown by Waelsch & Borek who found that α -keto- γ -methio-butyric acid, which results from enzymatic deamination of methionine (see above), is, in sharp contrast to the latter, readily hydrolyzed with formation of the mercaptan. The isolation from fermentation products of methionol, $\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}_2\text{OH}$, by Akabori, indicates another possible path of enzymatic degradation of methionine. Regarding the possibility of methionine being a biological precursor of the thiazole component of thiamin see page 200. The chemical and biological literature pertaining to methionine before 1938 has been reviewed in detail by Toennies (2).

Glutathione.—The isolation of glutathione from yeast has been simplified by Schroeder *et al.* by beginning with an acetone treatment of the yeast in order to liberate the compound. In a patented process Schoeller & Goebel claim improvement of the yield from 40 to 50 to more than 80 per cent by reducing losses caused by oxidation. This is achieved by precipitating, by organic solvents, an alkaline earth salt of the compound subsequent to its liberation from the cuprous salt. A procedure for the preparation of disulfide glutathione as a glassy product has been reported by Schöberl & Hornung.

The problem of the relative oxidizability of glutathione and ascorbic acid, brought to the fore by the studies of Hopkins & Morgan (1) and Kertesz is far from clarified. Braier states that in a slightly alkaline solution kept below 22° ascorbic acid is rapidly and irreversibly oxidized by air without affecting any glutathione present in the system, while Fujita & Numata (1) find that in the presence of ascorbic acid oxidase the vitamin is rapidly oxidized at 30° and pH 6 to 7 without affecting either cysteine or glutathione. It seems that much fundamental knowledge is still lacking regarding the oxidation-reduction aspects of glutathione chemistry. According to Shinohara & Padis, working at pH 5.2, Folin's phosphotungstic acid reagent is only very incompletely reduced by GSH. Schöberl & Krumey found that the extent of reduction increases with increasing pH until at pH 7.3 completeness of the reaction prevails. While from these findings it might be inferred that the redox potential of the glutathione system is higher than that of the cysteine system, the investigation of the same

reaction (at pH 5.7) by Kassell & Brand (1) points to a difference of kinetics rather than of potential as the cause of the different behavior. This would be in harmony with the experiments of Fruton & Clarke according to which the redox potential of the two systems, at pH 7, is substantially identical. Undoubtedly the difference in the acid dissociation constants of the thiol groups which is to be expected between a dicarboxylic-monoamino acid (GSH) and a monocarboxylic-monoamino acid (cysteine) is a factor in the velocity of the oxidation-reduction reactions (cf. Bersin & Steudel and Schöberl & Krumey) and their pH dependence. Kassell & Brand (1), and likewise Schöberl & Krumey, observed that even at pH 5.2 to 5.7 the reaction of GSSG with phosphotungstic acid, and also that of GSH, reaches prompt completion when sulfite is present. Possibly this striking acceleration is the result of the elimination (through reaction with sulfite) from the reaction equilibrium of the GSSG formed by the oxidizing action of the color reagent.

Considerable progress has been made in the development of practical analytical procedures for the determination of glutathione. Certain improvements of detail in their cadmium salt method (1) have been advanced by Binet & Weller (2). However, according to the critical experiments of Berenstein & Birkhauser and Fujita & Numata (1) the method seems likely to produce grossly erroneous results. It is unfortunate that under these conditions the large amount of work which has been carried out by means of this method, especially by French and Russian authors, on a variety of biological materials, must be considered of doubtful value. Corkill & Nelson have obtained good results by improving the ferricyanide method of Mason—which does not differentiate from cysteine—by a photometric procedure. Fujita & Numata (1, 2, 3, 4) have advanced carefully designed procedures for the determination of glutathione (plus cysteine) in tissue extracts by iodometric and colorimetric (nitroprusside) methods. They prove that reduction of the disulfide forms by zinc, magnesium, cyanide, or catalytic hydrogenation methods is not dependable, and use instead, with excellent results, hydrogen sulfide in the presence of mercuric acetate. Through separate determination of cysteine-cystine, by a methylene blue procedure (see p. 204), they obtained by the two methods glutathione values, which, except in certain organs (thymus, corpus luteum, testis) where the iodometric values are substantially higher, are in good agreement. The only glutathione method for which a claim of strict specificity seems justified is that of Woodward (1)

based as it is on an enzyme-activating property of the peptide. It is gratifying that a titrimetric procedure [Schroeder & Woodward (1)] has now been developed, which can take the place of the more specialized manometric technic, and furthermore, that disulfide glutathione has also been made available for this determination method by the creation of an electrolytic reduction procedure (Dohan & Woodward). For other aspects of the determination of glutathione the reader is referred to pages 202 to 206.

A considerable literature continues to appear on the physiological distribution of glutathione and its variations under different conditions. A critical discussion of these data, which to a large degree suffer from the uncertainty attached to the analytical methods employed, shall not be attempted here, especially since few new conclusions, beyond those outlined by du Vigneaud & Dyer in Volume V of this *Review*, seem to have emerged.

Woodward (2) demonstrated the hydrolysis of disulfide glutathione, with liberation of cystine, by blood serum through an enzyme action similar to, although much less powerful than, that previously observed in kidney tissue by Schroeder & Woodward (2). Of special interest is also the demonstration, by Ganapathy & Sastri (1), that papaya and similar fruits in the fresh state contain as much as 0.3 per cent of glutathione [determined by Woodward's (1) specific method] which in turn represents not more than 10 per cent of the total thiol groups (nitroprusside test) among which free cysteine (Fleming's specific method) is not present. The authors suggest that the cysteine-glutamic acid peptide found by Grassmann in commercial papain represents a decomposition product of the glutathione originally present.

Insulin.—A valuable reference work has been made available by Jensen's book on the chemistry and physiology of insulin. An interesting short review on the hypoglucemic activity of organic sulfur compounds and the role of sulfur in insulin has been contributed by Braun *et al.*

Recently Miller & du Vigneaud showed that more than 95 per cent of the sulfur of insulin could be accounted for by cystine when the hormone was hydrolyzed by a mixture of formic and hydrochloric acid while previously, with ordinary hydrochloric acid hydrolysis, it had been possible thus to identify only three-fourths of the total sulfur. These findings have now attained new significance through interesting observations of Sullivan & Hess which may prove to be important for the problem of the constitution of insulin. Sullivan &

Hess showed that recrystallization of insulin from a buffer system containing acetic acid, ammonia, and pyridine (du Vigneaud, Jensen & Wintersteiner) while leaving the total sulfur content of the compound unchanged, affects a part of the cystine components in such a way as to lead to a loss of sulfur in volatile form during ordinary hydrochloric acid hydrolysis. Such loss was not in evidence, however, when hydrolysis was performed by the formic-hydrochloric acid method. Furthermore, when the insulin was recrystallized from phosphate buffer in the presence of zinc ion (Scott), instead of by the above mentioned method, the resulting product showed no evidence of labilized cystine in either hydrochloric acid or formic-hydrochloric acid hydrolysis. While this difference in lability, of what seems to be a definite fraction of the cystine building blocks, showed no detectable counterpart in the physiological assay value, an interesting new avenue of approach to the study of the insulin architecture has undoubtedly been provided. In pursuit of their findings Hess & Sullivan (1) have reported that certain synthetic cystine peptides give lower cystine values after hydrochloric acid hydrolysis than after hydrolysis with formic-hydrochloric acid, while others do not. Sullivan *et al.* have also shown that, contrary to a report of Tropp, the polarographic method (see p. 205) yields the same cystine values as the Sullivan method in insulin hydrolysates.

Roberts, in the course of studies of proteins in liquid ammonia, found that when insulin remains dissolved in that solvent for twenty-four hours its hypoglucemic action is not affected, but that by the slightest reduction by sodium the action is completely obliterated. A very considerable enhancement of physiological action can be obtained by irradiation of insulin with Roentgen or ultraviolet rays, according to Ito. Vartiainen & Bastman reported a retardation effect, similar to that caused by protamine, resulting from the addition of arginine. Insulin preparations of delayed action, obtained by means of alums, have been described in a patent by Rosenthal & Kamlet. Sahyun, Nixon & Goodell demonstrated that small amounts of cobalt, nickel, or aluminum are similar to zinc in their capacity to retard the spontaneous deterioration of insulin preparations. Vogelenzang in a study of insulin preparations containing different metals, with regard to iodine consuming power and physiological activity found a definite relation between the two which, he hopes, will permit the development of a chemical method of assay.

Toxins.—The discussion between Slotta and Micheel concerning

the toxins of snake venoms has centered around the nature of the sulfur groups, and especially the presence or absence of essential disulfide linkages. Micheel & Schmitz (1) believe that different conclusions are largely due to disregard of the fact that the toxins of the two classes of poison snakes, the colubrides (cobra, etc.) and viperides (rattlesnake, etc.) are of different chemical nature, just as their physiological action is definitely different. Micheel & Bode have reached the conclusion that in the activity of cobra neurotoxin neither disulfide groups, nor, as had previously been suggested by Micheel, Dietrich & Bischoff, thiolactone or thiazolidine groups are concerned. This conclusion resulted from the finding that the rate of inactivation by sulfite decreases with increasing pH, contrary to the behavior of the sulfur groupings mentioned in their cleavage reactions with sulfite. Micheel & Bode emphasize that the evidence of Slotta, Forster & Fraenkel-Conrat—identifying the total sulfur as cysteine sulfur according to Sullivan—does not necessarily conflict with their conclusions as the cysteine may be present in the form of unknown thioether linkages which may be split under the conditions of protein hydrolysis.

As to the rattlesnake toxin, the beautifully crystallized protein isolated by Slotta & Fraenkel-Conrat and named crotoxin is, according to Ghosh & De, not a single compound but a mixture of at least two different proteins, since they succeeded in a partial separation of the hemolytic from the neurotoxic principle.

Keratins.—This class of proteins is of special interest from the point of view of this review because of the prominent role which sulfur compounds seem to play in their structure. Keratins are characterized by their insolubility, high resistance to enzymatic digestion, and common ectodermal origin (Block). Block has distinguished between the proteins of the epidermal appendages (hair, claws, feathers, etc.) which he calls eukeratins and which are characterized chemically by their containing the basic amino acids histidine, lysine, and arginine in the approximate ratio 1:4:12, and, on the other hand, pseudokeratins, which are chiefly represented by the proteins of the normal skin and the nervous system (neuroproteins), and in which the lysine to arginine ratio approaches unity. The sulfur content of most pseudokeratins lies between 1 and 3 per cent, at least one-half of which is present in the form of cystine (Block & Bolling). In the eukeratins the sulfur content tends to be higher (3 to 5 per cent), and nearly all of it (80 to 100 per cent) is present as cystine (Block *et al.*). While the true nature of normal epithelial keratin seems to

be still in doubt (cf. Block) it is of interest that Wilkerson & Tulane demonstrated in stratum corneum of human epidermis, obtained from cases of exfoliative dermatitis, that the total sulfur (1.1 per cent) is about equally divided between cystine and methionine. Considerable significance must be attached to the results obtained by Block & Lewis in an investigation of animal hairs. They found that in cow hair the percentage of the total sulfur which could be accounted for as cystine (by Sullivan method after hydrolysis as well as by the labile sulfur method of Zahnd & Clarke) consistently increased with the age of the animal (from below 80 per cent at three months to at least 99 per cent at five years). This finding is the more interesting as the values for total nitrogen, tyrosine, tryptophane, as well as total sulfur showed no significant variation, so that one might suspect a process of gradual substitution of cystine for methionine, or conversion of the latter into cystine.

It has long been recognized that individual keratins are not chemically homogeneous. Interesting evidence in this connection, which also shows that physical and chemical resistance of keratins is to a considerable extent related to mechanical structure, has been adduced by Routh & Lewis. They found that by mechanical grinding an increasing fraction of wool was rendered water soluble and that at the same time a notable degree of enzymatic digestibility resulted. However, the observation that grinding caused a lowering of the cystine content and appearance of sulfate ion indicated that not only mechanical breakdown but even chemical action of an oxidative nature occurred. Elsworth & Phillips have shown that the reaction of Clarke, $-S-S- + H_2SO_3 \rightarrow -SH + -SSO_3H$, is an important factor in the commercially customary bleaching of wool with sulfur dioxide or sulfite solution. By treatment with bisulfite ion under optimal conditions about one-third of the total sulfur could be made to react in this way. It could be shown that, in accordance with Clarke's findings, the S-sulfonate groups formed in the wool hydrolyze with the formation of sulfhydryl compounds and sulfuric acid on boiling in a strongly acid ($pH < 2$) medium, while at lower acidity ($pH 3$) the alternative cleavage, into sulfenic acid ($-SOH$) and sulfurous acid, prevails. Phillips found that the whiteness of the resulting wool was at a maximum when the bisulfite treatment was carried out at $pH 5$ coinciding exactly with the pH of optimal interaction of wool and bisulfite found by chemical analysis. Since the action of hydrogen peroxide, which is also used for wool bleaching, was shown by Smith

& Harris (also Rutherford & Harris) likewise to consist in an attack of disulfide bonds, through oxygenation, Phillips suggests that in complex peptide structures disulfide cross linkages may, owing to inherent strains, possess chromophoric character. Speakman & Coke have submitted evidence to show that another commercial process, "carroting," i.e., the treatment of furs with mercury salts, used for increasing their felting capacity, consists at ordinary temperatures largely in reactions with basic side chains and possibly peptide imino groups, while the interaction of disulfide groups with mercuric chloride ($\rightarrow \text{RSCl} + \text{RSHgCl}$) begins to play a role above 40° . Other valuable contributions to the chemistry of wool, only a few of which can be mentioned in this review, have continued to come from Harris and associates. Harris & Rutherford showed that the amount of actual acidic groups in wool protein can be determined by exposure to alkali when a correction is made for the alkali consumed through reaction with disulfide groups. This correction is obtained by determination of sulfide ion formed according to the sequence of reactions, $\text{RCH}_2\text{SSCH}_2\text{R} + 2 \text{OH}^- \rightarrow \text{RCH}_2\text{S}^- + \text{RCH}_2\text{SO}^- + \text{H}_2\text{O}$, $\text{RCH}_2\text{SO}^- \rightarrow \text{RCHO} + \text{H}^+ + \text{S}^{=}$. Formation of hydrogen sulfide, presumably by a similar mechanism, was shown to take place in the photochemical deterioration of wool (Harris & Smith).

Biological occurrence and functions.—From the seeds of a Canadian weed C. Y. Hopkins has obtained 2-mercapto-5,5-dimethyloxazoline,

line, $(\text{CH}_3)_2\text{C} \cdot \text{CH}_2 \cdot \text{N} : \text{C}(\text{SH}) \cdot \text{O}$, which presumably originates from a mustard oil glucoside derived from β -methylallyl isothiocyanate. Addition of water to the latter and ring closure may result in the formation of the mercapto-oxazoline.

An interesting contribution is the isolation, by Theorell, of *l*-cystine from porphyrin-*c*, the metal-free degradation product of the important iron-containing respiratory carrier cytochrome-*c*. On the basis of the absence of sulfhydryl or disulfide reactions Theorell assumes that two cysteine molecules are attached as thioether side chains to the porphyrin nucleus so that a genesis of porphyrin-*c* through addition of cysteine to the vinyl side chains of protoporphyrin can be postulated. If Theorell's proposed structure is confirmed porphyrin-*c* would seem to represent the first known example (unless djenkolic acid is so considered) of the physiological occurrence of cysteine in thioether linkage; a type of linkage which Micheel in his work on snake toxins has suggested as being worthy of consideration in protein analysis.

In further confirmation of Hammett's theory of the natural function of sulfhydryl groups in cell division Hammett & Chapman (2, 3) have demonstrated positive correlation between proliferative activity and sulfhydryl concentration in the cells of sprouting beans and *Obelia geniculata* while the correlation between cell elongation and sulfhydryl concentration was negative. The effectiveness of thiocyanate in breaking the dormancy of grass plants and stimulating their growth has been demonstrated by Shepherd. An interesting review on the biological role of sulfhydryl groups is that of Wels which centers around the author's theory, and provides evidence for it, that the photochemical liberation and activation of sulfhydryl groups in the skin are important factors in the beneficial effects of irradiation. Eagle has found that sulfhydryl compounds (cysteine, glutathione, etc.) can greatly diminish the antipirochetal action of arspenamine and similar organic compounds of bismuth and mercury. He believes this to be due to compound formation and considers that the antipirochetal action of the compounds may be due to a chemical affinity to sulfhydryl groups in the organism. Gersdorff in a toxicity study of cresol and thiocresol on goldfish has found that the thiol compound is about four times as toxic as the hydroxyl compound.

Protein denaturation.—The term denaturation^a is used to describe transformations of proteins which are caused by physical or chemical influences and which become evident primarily through changed physical properties such as decreased solubility, changed viscosity, etc. That these changes are the expressions of, or associated with, definable alterations of a chemical nature is so far chiefly shown by the appearance upon denaturation of reactive sulfhydryl groups. It has been known for some time that thiol groups are thus "liberated" by concentrated solutions of urea, and a study of this phenomenon has now been entered into by Greenstein (1, 2, 3). This investigation seems to represent a promising approach to the fundamental problem of the true nature of the cysteine components in protein molecules because one of its first results has been the emergence of the question: What type (or types) of linkage will generate (or unmask) sulfhydryl groups under the influence of such media as concentrated aqueous urea solutions? The possibility that the process is a simple hydrolysis or reduction of disulphide groups has been tentatively dismissed because the reagents in question remained without any effect on syn-

^a For an adequate discussion of the subject in its entirety cf. Anson (1).

thetic disulfide (cystine) peptides. For elucidation of the mechanism Greenstein has, however, begun by studying the effect of variations of the reagent, and it was found that among urea and various related compounds guanidine in the form of neutral salts, i.e. as guanidonium ion, was the most effective compound. It further became clear that anions are essential participants in the process. Thus, while among guanidine salts the hydrochloride, thiocyanate, hydrobromide, nitrate, and hydroiodide are increasingly effective, sulfate, carbonate, or acetate are totally ineffective. The more favorably situated anions, e.g. thiocyanate and iodide, were even found to have a sulfhydryl liberating effect as potassium salts. A maximum and constant sulfhydryl value was generally obtained, when guanidine hydrochloride was used, with a concentration of the latter of about 5 *M* or above. The constant maximum value obtained when urea in increasing concentrations was used was in all cases lower than the value obtained with guanidine. The complexity of these phenomena becomes further evident when the responses of different substrates are considered. The thiol groups which could be mobilized by guanidine hydrochloride represent (approximately) in amandin none, in excelsin one-ninth, in edestin one-third, in egg albumin two-thirds, in globin three-fourths, and in myosin and tobacco mosaic virus protein (Stanley & Lauffer) practically all of the alkali labile (i.e. presumably cysteine) sulfur of the protein. The case of myosin (Edsall, Greenstein & Mehl) is especially interesting. Its double refraction of flow, an expression of its pronounced molecular asymmetry, is diminished or abolished by various denaturing agents. However, no systematic correlation between the action of different agents on double refraction and appearance of sulfhydryl groups could be detected. On the basis of this and other evidence which cannot be cited here Greenstein (2) has concluded that "the number of sulfhydryl groups liberated, which may be taken as a measure of the change in protein configuration, is clearly different according to the nature and the concentration of the individual denaturing agent," but also "that no obvious relation exists between the differences of molecular weight in different solvents and the appearance of sulfhydryl groups." The new problems raised are thus numerous and further results will be awaited with interest. As to the question of whether the sulfhydryl liberating reaction represents generation of sulfhydryl groups by the cleavage of bonds between sulfur and other atoms or rather an "unmasking" of existing sulfhydryl groups as a result of a general opening of the molecular struc-

ture, a new observation of Anson (2) favoring the latter view appears significant. Anson found that the sulfhydryl reaction which can be elicited by denaturation is diminished or abolished when the protein was, preceding denaturation, subjected to the mild action of iodoacetamide or iodine, reagents which are known to eliminate sulfhydryl groups by condensation or oxidation.

Enzyme and hormone action.—Many-sided advances pertaining to the participation of sulfur groups in these functions have been discussed in last year's review by Medes. The outstanding developments in this field that have since come to light relate to the nature of papain. Hellerman's review presented the evidence upon which is based the theory that the active enzyme contains sulfhydryl groups the reversible oxidation of which to disulfide inactivates the enzyme. Now doubts regarding the unqualified acceptability of this view have appeared. The complete absence of a nitroprusside test, either before or after hydrogen cyanide activation of a highly purified papain preparation, which was reported by Okumura, may in the case of a protein molecule not justify the conclusion that sulfhydryl groups do not exist (cf. p. 216), but experiments of Ganapathy & Sastri (2) show that sulfhydryl groups, if they are present, are essential only for the pepsinase activity while apparently unnecessary for the gelatinase activity of the enzyme. In addition they conclude that another group is present which is essential for both activities and which is, unlike sulfhydryl, not inactivated by maleic acid, while its function is completely annulled by a very small amount ($3 \times 10^{-6} M$) of iodoacetate. Even more important, because it was carried out on crystalline papain, purified to constant activity, appears to be an investigation of Balls & Lineweaver. They found that oxidation by porphyrindin has little or no effect on the activity of the enzyme although iodoacetate, in the amount of only one molecule per papain molecule, causes complete inactivation. They conclude that groups other than sulfhydryl are responsible for the reduction of porphyrindin which does take place, and as to the actual role of sulfhydryl groups, they suspend judgment pending further investigation. With regard to the action of iodoacetate Ganapathy & Sastri (2) concluded from their own experiments that in enzyme work it has other effects besides binding sulfhydryl groups. This view⁴ was also reached by Haag & Bolomey, who in a study of alcoholic fermentation by zymase, found inactiva-

⁴ Cf. also *Ann. Rev. Biochem.*, **8**, 203 (1939).

tion by less than 0.001 *M* iodoacetate which did not involve sulfhydryl groups. It would seem that in appraising these phenomena the observation of Anson on the ability of iodoacetate to forestall the "liberation" of sulfhydryl groups upon denaturation (see p. 216) deserves consideration.

Maschmann has described proteinases and peptidases of anaerobic bacteria which are activated by ferrous iron, and, if iron is present, also by cysteine or ascorbic acid. In these cases the activating function of the thiol compound presumably consists in protecting the ferrous iron against oxidation. Different mechanisms again will have to be postulated for the case of pancreatic esterase reported by Cedran-golo. Here the synthetic (on oleic acid and glycerol) and hydrolytic (on olive oil) activities are inhibited by cysteine or ferric ion, while cystine and ferrous ion have an enhancing effect. However, an enzyme which in its response to different reagents fully complies with the specifications for an active sulfhydryl and inactive disulfide form is the cholinesterase (which hydrolyzes acetylcholine into acetic acid and choline) reported on by Nachmansohn & Lederer.

In regard to respiratory enzyme systems Chaix & Fromageot showed, on propionic bacteria, that cysteine and hydrogen sulfide suppress the Pasteur effect, i.e. they prevent the oxidative system from prevailing, in aerobiosis, over the fermentative system, presumably by retaining the latter in the reduced form. Euler & Hellström have reported data which are in harmony with the suggestion of Hopkins & Morgan that succinodehydrogenase may be a sulfhydryl-active—disulfide-inactive enzyme. Similar experiments indicating that the activity depends on free sulfhydryl groups have been shown for glyceraldehyde dehydrogenase by Rapkine & Trpinac.

While the examples of enzymes in which reversibly oxidizable sulfur seems to play a role have become numerous the chief example of a similar function in a biocatalytically active substance of hormonal nature is insulin. It would be both interesting and suggestive of interrelated significance if future work were to continue to show that among biocatalysts containing functional sulfur the enzymes tend to depend for their activity on the sulfhydryl state while the disulfide stage is the active one among hormones. That the latter may be true for hormonal principles other than insulin is suggested by an investigation, by Fraenkel-Conrat *et al.*, of gonadotropic hormones. They found that gonadotropic preparations obtained from the pituitary as well as from female menopause and normal male urine were inacti-

vated by incubation with cysteine. They were able by the response to cysteine to differentiate these gonadotropic hormones, all presumably of pituitary origin, from those of placental origin (found in pregnancy urines) which remained uninfluenced by cysteine. In another investigation (Meamber *et al.*) they were able to differentiate the growth promoting hormone of the pituitary from the associated lactogenic, thyrotropic and gonadotropic principles because all but the former were inactivated by cysteine.

In connection with biocatalytic questions a model experiment, reported by Kather, seems of interest, because it describes a system where a metal-mercaptide is the active catalyst. While the numerous presumed sulfhydryl enzyme systems are inactivated by mercaptide formation and reactivated by removal of the metal ion (e.g. by hydrogen cyanide), some systems have been described where conversely a mercaptide can be assumed as the active form (cf. Hellerman). Kather shows that the sulfhydryl groups liberated photochemically in egg white combine with copper ion to form a complex which catalyzes the oxidation of unsaturated fatty acids. The catalytic action is not influenced by iodoacetate but is inhibited by cyanide.

Metabolism.—Additional contributions have appeared defining the position of different derivatives of the sulfur amino acids as to their capacity of being metabolically utilized for support of growth. The non-availability of certain N-substitution products in young rats either for growth support or for degradation of their sulfur to sulfate, was demonstrated by Jen & Lewis for the dibenzoyl and betaine derivatives of *l*-cystine. Andrews showed similar non-availability for cysteic acid and hydantoin derivatives. Bennett in a further study with graded quantities found that for the support of growth two molecules of *l*-cysteine or two molecules of *l*-methionine or *three* molecules of *l*-cystine disulfoxide are equivalent to one molecule of *l*-cystine. Her data further indicate definite differences in the metabolic responses to *dl*- and *l*-methionine. The latter appeared quantitatively more effective than the former in promoting growth and, besides, produced a striking improvement in the efficiency of food utilization.

Very interesting advances have been made concerning the metabolic relations between methionine and cystine. The strong implication of earlier findings that the former can be biologically converted into the latter has now been given the support of direct evidence. Beach & White by determining the cystine content of entire animals showed that rats raised on a diet almost devoid of cystine accumulated sub-

stantially more cystine than could be accounted for by the small amount taken in. That the synthesized cystine actually originates from methionine was shown by Tarver & Schmidt when they fed animals methionine containing radioactive sulfur (S^{35}) and subsequently isolated from the animals cystine containing the radioactive isotope. However, Madden *et al.* showed that in the regeneration of plasma protein in dogs, after artificial depletion, dietary methionine cannot substitute for cystine. Another, related, aspect of the problem of the biochemical position of methionine has been significantly illuminated by simultaneous investigations of Rose & Rice and of du Vigneaud, Dyer & Kies which brought out the fact that, contrary to previous conclusions, homocystine or homocysteine cannot by itself replace methionine in the biosynthesis of young rats. Previous contrary findings were shown to be due to the presence, in the vitamin concentrates used, of a supplementing factor which rendered homocystine adequate. When instead of crude B-extracts, pure compounds (thiamine, riboflavin, nicotinic acid) were used the non-equivalence of homocystine and methionine became apparent. Subsequent investigation of this unexpected situation by du Vigneaud, Chandler, *et al.* revealed that the effectiveness of homocystine as a substitute for methionine depends on the presence in the diet of a certain quaternary methyl ammonium structure exemplified by choline and betaine. It will be interesting to see if this supplementation will be found equally necessary for the biosynthesis of both methionine and cystine. Other observations in this sphere, which for the time being add to the complexity of the picture, are the findings of Singal & Eckstein in connection with the antagonistic effect of methionine and cystine on the fat metabolism of the liver, that here homocystine does not act like methionine but like cystine, and the work of du Vigneaud, Wood & Irish which produced evidence that metabolic degradation of S-benzylhomocysteine to the cysteine stage does not occur. The same result was obtained by Stekol (2) in connection with his detoxication studies. Excretion of S-benzylhomocysteine as the acetyl derivative (of the *l*-form after feeding of either *d*- or *l*-form of the non-acetylated compound, according to du Vigneaud, Wood & Irish) is in harmony with the theory of Knoop on the role of N-acetylation of amino acids in normal metabolism, which recently was reestablished by du Vigneaud & Irish. The same theory fully explains the general occurrence of the acetyl group in the detoxication products known as mercapturic acids. Another interesting result of Stekol's (1) studies

is the finding that inhibition of growth caused by ingestion of bromobenzene or naphthalene was counteracted by the addition of glutathione to the diet. Since he had previously found that, contrary to the effect of cysteine or cystine, the synthesis of mercapturic acids was not increased by glutathione he concludes that utilization of glutathione for growth purposes may take place without cleavage of the peptide. This observation also lends support to Stekol's previous conclusion that the cysteine moiety of the mercapturic acids excreted after the ingestion of aromatic substances is of endogenous origin.

That the biological path of a sulfhydryl compound can be radically different from that of the corresponding disulfide compound, as was first emphasized by Brand, was again shown to be true in another metabolic function when Virtue & Doster-Virtue (2) found that homocysteine, unlike homocystine, can be utilized in the synthesis of taurocholic acid. The study of the mechanism of taurocholic acid formation by the authors named was further advanced by their (1) observations that cystine disulfoxide, cysteine sulfinic acid, and cysteic acid are increasingly effective in causing taurocholic acid formation. They conclude, from this and from their previous observation of the ineffectiveness of cystamine, that in the biological conversion of cystine to taurine oxidation to the sulfonic acid precedes decarboxylation.

The metabolic studies of the last several years on sulfur compounds have revealed the physiological occurrence of numerous reactions (formation of sulfate, taurocholic acid, the different metabolic paths of cysteine and cystine, conversion of methionine to cystine, etc.) which from the standpoint of *in vitro* chemistry are unusual, and beginnings are now being made in the uncovering of the enzyme mechanisms at work. Fromageot and associates have studied the desulfuration of cysteine and other thiol compounds by bacterial enzyme systems. *B. coli* was shown [Fromageot & Moubasher (1)] to possess a system that removes the sulfur of cysteine and cystine (*d*- and *l*- with equal ease) as hydrogen sulfide, quantitatively and without deamination or decarboxylation. Thiolactate and glutathione are similarly decomposed while thiourea, methionine, or taurine are not attacked. The presence of compounds like formate, lactate, fumarate, aspartate, or glutamate, apparently as hydrogen donors, is necessary in the case of cysteine, but not in the cases of glutathione, possibly because of the presence of the glutamate radical in the molecule [Fromageot & Moubasher (2)], and of thiolactate. In the latter case

the process was shown to be a hydrolytic one (Fromageot, Moubasher & Desnuelle) involving formation of lactate and hydrogen sulfide, while cysteine was shown (Fromageot & Desnuelle) to undergo a reductive process in which alanine is formed. Clark & Tanner studied numerous strains of thermophilic bacteria for their ability to produce hydrogen sulfide from peptones. This was found to vary with different bacteria and also to depend on the peptone used. However, no strain was found that could produce hydrogen sulfide from methionine. Another mechanism, capable of effecting a methylating cleavage (formation of sulfhydryl and methio derivatives) of dibutyl- and diamyldisulfide, was demonstrated in *Penicillium brevicaulis* by Blackburn & Challenger. The compounds studied are of interest because of their known occurrence in animals of the skunk family.

An outstanding contribution is a study by Medes which attacks the practically uncharted field of the enzyme chemistry of mammalian sulfur metabolism. By the tissue slice technic Medes has obtained evidence for the presence, in rat liver, of at least four different enzyme systems concerned with the degradation of cysteine and its oxidation products. One process, which is inhibited by cyanide, is the oxidation of cysteine to the disulfide stage. The enzyme responsible for this reaction is thermostable and was shown to be identical with cytochrome oxidase. Another system, which is thermolabile, causes oxidation of cysteine as well as cystine (whether or not by way of previous reduction to cysteine is not as yet clear) to the sulfonic acid stage. Most interesting is the fact that two alternative sulfate producing systems could be identified. A highly labile system, easily inactivated, especially by dialysis, oxidizes cysteine to sulfate. In order to enable cystine to serve as a substrate in this process its preliminary reduction, presumably by another enzymic factor (cf. Pirie) appears necessary. The fourth enzyme was termed sulfinic acid oxidase. It is a highly active thermolabile principle, distinguished from the preceding one chiefly by its stability against dialysis, which produces sulfate from cysteine sulfinic acid. Speculation as to the interlacing of the different pathways is enticing especially since the sulfinic acid appears to represent an intermediate stage in the process promoted by the second, sulfonic acid forming enzyme. Experiments of Bernheim & Bernheim show that certain metals, as titanium, manganese, and cobalt, which are normal tissue constituents may have regulatory functions at the metabolic crossroads. They found that titanium favors the oxidation of cysteine to cystine, but strongly inhibits the

oxidation to the sulfonic acid stage which is promoted by a thermolabile system, presumably identical with the second enzyme of Medes. Similar inhibition was shown for manganese and cobalt.

The year has also brought forth a pioneering investigation concerning the sulfur metabolism of higher plants. Mothes in an extensive paper describes results obtained by fractional analysis (protein sulfur, free and esterified sulfate, partially oxidized and "neutral" sulfur) of the different anatomical parts of leaf plants, grown under different conditions. It appears that reduction of sulfate with synthesis of organic sulfur compounds as well as their oxidation back to sulfate are actively taking place. Young active organs are rich in neutral sulfur (glutathione, etc.) and the sulfate content generally increases with age. Sulfate is viewed as a readily transportable sulfur reserve without important physiological function of its own. The inert nature of sulfate in the animal organism, by contrast, is demonstrated by the fact, shown by Aten & Hevesy, that the greatest part of injected sulfate labelled by isotopic oxygen leaves the body unchanged.

In this review papers which are primarily concerned with general nutrition or general protein metabolism have been omitted from consideration.

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FAT METABOLISM

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Absorption.—The classic studies of Munk and much subsequent work have led to the view that after hydrolysis some 70 per cent of food fat is absorbed through the lymphatic system while the remainder passes directly into the capillaries. Evidence, however, is lacking as to why some fat should thus be made directly available to the liver through the portal circulation when the bulk of the fat passes into the systemic blood; nor is knowledge available as to the factors which determine which fraction of the fat is absorbed into the capillaries and which into the lacteals. Careful investigations which may have an important bearing not only on this aspect but also on the mechanism of absorption have been carried out by Frazer and his colleagues (1, 2, 3, 4, 5). After satisfying themselves that the particles seen in blood under dark-ground illumination are essentially glycerides with probably an adsorbed globulin layer, they established the fact that the chylomicron count runs parallel to the blood glyceride content. They then observed that administration of olive oil caused an increase in the particle count in the systemic blood but no increase in the portal blood; in contrast, oleic acid caused an increase in the chylomicron count of the portal blood but no increase in that of the systemic blood. From these and other results they have advanced the theory that hydrolysis of fat is not necessary for its absorption and that unhydrolysed fat passes into the lymphatic system while the hydrolysed fat passes directly to the liver. In opposition to extensive previous evidence, these findings reopen once again the much debated question of the occurrence of particulate absorption; if they are substantiated, it may be found that the liver has a direct call on the unsaturated acids present in food fat since it is possible that liberation of unsaturated acids from glycerides may be more readily accomplished by lipase than is that of saturated fatty acids of the same chain length. Further work along these lines will be awaited with much interest.

Fat absorption in certain fishes is a subject which has also received study. The finding of much vitamin A and A₂ in the intestines of certain fish (6) was followed by histological study of the intestine

of the halibut (7); later it was found that 95 per cent of the vitamin A of halibut intestinal oil is present as esters (8) while results of the analysis of the oil show that the fatty acids with which vitamin A is esterified occur in proportions similar to those in the main fat fraction; in addition the occurrence of much free fatty acid in oil prepared from fish immediately after death was observed (9). Reviewing all the data obtained, Morton & Lovern suggest the interesting hypothesis that vitamin A esters are concerned in fat absorption in certain fish. The fact that the *tunica propria* contains as much as 60 to 70 per cent of vitamin A esters is certainly of great interest, but clearly much more definite evidence will be required to substantiate this hypothesis.

The part played by phosphorylation in fat absorption was reviewed last year; there it was shown that evidence was accumulating which was opposed to the view of Verzár that the adrenal plays an important part in fat absorption because of its supposed specific function in causing phosphorylation. Further similar evidence is now available, for it has been reported that adrenalectomy in rats has no effect on the rate of absorption either of the methyl esters of the fatty acids of corn oil or of corn oil itself (10).

Detailed studies have been made of the fecal excretion of individual lipoids by healthy children (11).

The liver.—The problem of fat metabolism in the liver which has been so extensively studied in the last few years is still being widely investigated. Further conditions in which fatty infiltration into the liver occurs have been added to the already long list. Fatty livers are now reported to occur in mice fed on cocaine (12) and in guinea pigs receiving a scorbutogenic diet (13).

The action of hormones on liver fat remains a much-debated topic. In confirmation of the findings of certain previous workers (15, 16, 17), but in contrast to those of Dragstedt *et al.* (18), Montgomery *et al.* (19) have further investigated the question of the part which the pancreas plays in fat metabolism. Careful ligation of the pancreatic ducts in dogs caused the occurrence of fatty livers irrespective of gains or losses in body weight; raw pancreas added to the diet prevented this abnormal fat deposition. However, as the authors point out, these results do not yet establish whether the agent which is active in preventing fatty livers is contained in pancreatic juice or whether the pancreas secretes a hormone, since atrophy of the gland results from ligation of the ducts. That adrenalectomy prevents fat

infiltration into the liver has again been demonstrated (20), while it is also claimed that injection of epinephrine produces a fall in blood fat with increase in liver lipids (21). Daily observations have been made on the effects of 70 per cent hepatectomy on the individual lipid constituents of the liver. The marked increase in the different constituents after twenty-four hours was followed by subsequent variable decreases from the new high level, but after seven days all the constituents studied had decreased although they had not reached the control levels (22).

The action of various toxic agents on fat metabolism in the liver has been studied and it is suggested that the results of carbon tetrachloride poisoning are due to interference with the ability of the liver to desaturate fatty acids (23), while liver injury caused by chloroform (24) and alcohol (25) has been discussed in the light of the nature of the diet.

The factors which prevent fat accumulation in the liver have also been further studied. Firstly, Best *et al.* (26) have added to the conditions in which choline is found to exert an effect by their studies on carbon tetrachloride poisoning in rats. Secondly, Platt (27) has reported further studies on the action of compounds related to choline. It had previously been found in the Liverpool laboratories that synthetic homocholine was more effective than choline itself and that substitution of the methyl groups by ethyl groups decreased the activity, while the tripropyl compound was without action; the new results show that choline methyl ether and tetra- β -hydroxyethyl ammonium chloride also have no action. These results confirm the necessity for the presence both of the alkyl groups and of the free hydroxyl group, the latter finding being explained if choline acts by accelerating lecithin synthesis. In confirmation of the findings of Best & Huntsman (28) and of Welch & Welch (29), betaine was found to possess activity, which was estimated to be 30 per cent of that of choline; colamine and creatine were without action. Thirdly, further information has been obtained concerning the preventive action on fat deposition which is exerted by certain proteins and protein constituents; to the classification of various proteins according to their lipotropic activities (30) has been added a finding by Singal & Eckstein (31) with respect to arachin. This protein, which is deficient in methionine, was found to be without lipotropic action, although the addition of small amounts of methionine rendered it as active as caseinogen itself; in contrast, the addition of cystine greatly increased

the already high level of fat caused by a diet containing arachin as the sole protein; it was found further that homocystine behaved in the same way as cystine. It has also been reported that tyrosine has some action in preventing fat deposition, and that serine, lysine, aspartic acid, phenylalanine, alanine, proline, hydroxyproline, histidine, valine, leucine, and arginine are without action (32).

This is further confirmation of the view that cystine and methionine are antagonistic in respect to their lipotropic activities: small amounts of cystine increase the liver fat content and small amounts of methionine decrease it; this substantiates previous findings (33, 34) and the report concerning homocystine adds greater interest to the means whereby these sulphur-containing amino acids exert their effects. It seems unlikely that they are involved in phospholipid metabolism, although du Vigneaud (35) has suggested, from his findings on the ability of homocystine with choline to replace methionine in growth experiments on rats, that possibly the action of methionine in fatty liver prevention is due to its providing methyl groups for the synthesis of choline from an unknown precursor, and possibly betaine itself might act in this way. On the other hand, the first step in methionine breakdown is supposed to be its conversion to homocystine which is now shown to increase fat deposition, a finding which makes this suggestion less likely. In any case the antagonistic action of cystine and methionine in the fatty liver problem is of great interest in view of the fact that these amino acids have hitherto been believed to pursue a common metabolic path. Further work is clearly necessary, but the reviewer feels that it is unlikely that the action of these amino acids will be found to be related directly to that of choline.

A further contribution to the problem of the possible conversion of fat to carbohydrate in the liver is provided in a preliminary note by Stewart & Thompson who, using the liver slice technique, observed an increase in carbohydrate which appeared to correspond to a decrease in the fat content of rat liver (36).

Phospholipids.—The fruitful discovery of Best and his colleagues at Toronto that choline was effective in preventing fat deposition in the liver has been interpreted from the start as meaning that choline exerted its effect by enabling lecithin synthesis to occur. Such an hypothesis followed readily from the conception of Leathes, supported by the subsequent work of Bloor and more recently by much interesting evidence from Sinclair, that lecithin plays an important part in the metabolism of fatty acids. It was supported too by the fact that

all the active analogues of choline have their alcoholic hydroxyl group free or in a readily available form (37, 27) and by the demonstration of Welch & Welch (38) that arsenocholine, which is also active, could be found in the lecithins prepared from animals to which it was administered.

While the earlier conceptions of the French school that the phospholipids of tissues are constant both in amount and composition has been modified as a result of much work which shows that the fatty acid constituents of phospholipids may react readily in certain conditions to changes in the nature of dietary fatty acids, the conception of the constancy of the amount of phospholipids in tissues has largely remained. For instance, even in studies concerned with livers that are intensely fatty it has been shown that the amount of phospholipid remains unchanged whether the liver be fatty or normal, or whether choline be administered or not (39), a finding now extended to the fatty livers of forcibly fed geese (40) and of mice receiving cocaine (12).

This difficulty has now been removed by the use of P^{32} by Perlman & Chaikoff who showed that the administration of choline markedly accelerates phospholipid turnover in the liver, the degree of acceleration being related to the amount of choline administered (41). By the same method these authors have also shown that betaine has a similar though less pronounced effect (42), an observation in agreement with the fact that betaine is less active than choline in preventing fat deposition. Betaine may yet fit into the phospholipid hypothesis of choline action, for Mann & Quastel (43) showed that liver slices can oxidise choline to betaine aldehyde, a change which may possibly be found to be reversible. Interesting also is the finding that the feeding of rats with cholesterol for only thirty hours decreased the phospholipid turnover in the liver, an excellent demonstration of the so-called cholesterol-lecithin antagonism, and an explanation of the production of fatty livers by dietary cholesterol; worthy of note in this same work is the fact that the acceleration in phospholipid turnover caused by choline feeding was greater in the livers of cholesterol-fed rats than in those of fat-fed rats (44).

All these experiments clearly demonstrate for the first time that the administration of choline or betaine to rats causes an increased rate of turnover of phospholipid in the liver. In this connection it is of interest to mention the work of Cavanagh & Raper (45), who studied the rate of turnover of phospholipids of the liver, kidney,

brain, and blood of rats from six to twenty-four hours after feeding with fat containing deuterium; their results also illustrate the rapidity of transfer and the fact that the liver phospholipids may play a very active part in fat metabolism. This work with isotopes, together with much of that of Sinclair, has shown that the observed constancy in tissue-phospholipid content under a variety of conditions, often extreme, has tended to be misinterpreted by some workers. It must be borne in mind, however, that these demonstrations of a rapid turnover of phospholipid in the liver and other tissues raise the problem of the fate of the newly-formed molecules since accumulation, if any, is only transient.

Much further work involving the use of radioactive phosphorus as an indicator of phospholipid metabolism has been carried out. Hevesy, whose lecture to the Chemical Society, London, reviews the advances made by this technique (46), has shown by studies with incubated hen's eggs injected with radioactive phosphorus compounds that the embryo obtains its phospholipids and other organic phosphorus compounds by synthesis from inorganic phosphate, and that it does not utilise the preformed compounds of the yolk or white (47). Studies on blood phospholipids have shown that liver is outstanding in its rate of uptake of radioactive phospholipid added to blood. Comparison of the results of experiments of this type with those in which synthesis of radioactive phospholipid after injection of active sodium phosphate was studied, shows that while both the rates of synthesis and exchange are high in liver, they are low in muscle. In kidney, synthesis is rapid but exchange slow (48). Blood derives its phospholipid from that synthesised in the various organs; the rate of replacement of the phospholipids of the red corpuscles is, as a whole, extremely slow, and is in contrast to that of the plasma phospholipids which are much more rapidly replaced (49, 50). Apart from the uptake of intravenously injected phospholipid by the liver, the spleen and lungs were also found to play a part in its removal from the blood; hydrolysis of the injected phospholipid caused the appearance of active phosphorus in the muscle, gastrointestinal tract, bones, and excreta at the expense of that of the spleen and liver (51). There is now much evidence, obtained both by experiments with radioactive phosphate and by other means, to show that the liver, kidney, and intestine are very active in phospholipid metabolism and therefore the demonstration by the Warburg technique that all these tissues are able to synthesise phospholipids is of importance (52).

While recent work has clearly shown that a considerable part of the phospholipids of certain tissues, such as liver, are intermediaries in fat metabolism, it is far from clear whether lecithin alone is involved. Sinclair (53), using his elaidic acid technique and separating the phospholipids of liver and muscle into lecithins and kephalins, found a somewhat greater rate of turnover in the lecithins of both tissues; while the rate of turnover of the liver lecithins was definitely more rapid than that of the kephalins, the rapid rate of turnover of the latter led Sinclair to the conclusion that kephalins as well as lecithins are intermediaries in fat metabolism. Using P^{32} , Chargaff has reported results for the lecithins and kephalins of intestinal tract, liver, brain, and carcass. Only in the intestine and to a lesser extent in the liver did the lecithins contain more P^{32} than the kephalins twenty-four hours after the feeding of rats; while the brain and carcass showed a much lower concentration of active phospholipid, the amounts of lecithin and kephalin undergoing change were similar in these tissues (54). Whether both lecithins and kephalins and even sphingomyelins are all active in fat metabolism therefore remains still undecided, although it is noteworthy in this connection that colamine is without action on liver fat. Studies of the phospholipid turnover of tumours by Chaikoff *et al.* (55) have revealed that it resembles that of the more active tissues such as liver, kidney, or intestine rather than the less active ones such as brain. The ready availability of P^{32} will doubtless lead to its extended use in phospholipid studies. At the present time much of the work has not been fully reported and it is difficult to assess its importance until more detailed information is available and wider studies have been made. Speaking generally, however, the use of P^{32} so far has provided satisfying and, in some cases, detailed confirmation by a very delicate method of findings which have already been largely established by other means, or surmised on indirect evidence.

The part played by phospholipids in blood clotting mechanisms continues to be investigated. Leathes & Mellanby (56) report the isolation of a thrombokinase from brain tissue and daboia venom which converts prothrombase into thrombase and which appears to be nonlipoid in nature. In contrast to the accepted view, they find that lecithin increases the activity of thrombokinase preparations, whereas kephalin has no such action and may even retard the formation of thrombase. On the other hand, Chargaff (57) has extended his studies of the thromboplastic activity of kephalin and finds, in accordance

with the accepted view, that a lysophospholipid preparation containing about 30 per cent lysokephalin lacks any influence on the blood clotting mechanism. Curiously enough, snake venoms were without action on pure samples of kephalin, and lysokephalin could only be formed by acting upon egg yolks.

Vitamins.—Since there is an increasing volume of work which implies that the vitamins B are probably involved in general fat metabolism, it is appropriate to discuss the present position from this point of view. Attacks on the problem of the part played by vitamin B₁ have come from three directions. Firstly, further results have been reported on the now accepted thiamine-sparing action of fat. Elvehjem *et al.* (58, 59) have shown that increase of the fat content of a low fat diet to 57 per cent by isocaloric replacement of sugar reduces the thiamine requirement of dogs to one-third of that necessary on the low fat diet (58). Symptoms of thiamine deficiency in rats were alleviated by isocaloric substitution of the carbohydrate by a variety of natural fats and also by synthetic tricaproin and triacetin; in this work studies of the thiamine content of the tissues were also made (59). That the decreased requirement of thiamine on diets high in fat was not concerned with the presence of traces of vitamin in lard has also been demonstrated (60).

Secondly, further evidence has been obtained that thiamine is concerned in the synthesis of fat from carbohydrate, for the original results of Whipple & Church (61, 62) on the rat, later confirmed by McHenry & Gavin (63), have now been extended to the pigeon (64). Thirdly, the conclusion of McHenry (65) that thiamine administration to rats receiving a low choline diet causes fatty livers has been confirmed for rats on a thiamine-deficient diet and extended to the chick (66), although in neither case was a preventive action of choline observed. If these three findings—the thiamine-sparing action of fat, a specific rôle of thiamine in causing the synthesis of fat from carbohydrate, and the fact that thiamine will, under certain conditions, cause fatty livers—be accepted at their face value, it is of interest to speculate how they may be correlated. Three points are worthy of mention. Firstly, synthesis of fat from carbohydrate has always been assumed to take place through a simple carbohydrate intermediary such as pyruvic acid, and it is now proven that thiamine is concerned in the metabolism of this substance. Secondly, other substances previously studied which increase the amount of fat in the liver are believed to act by mobilising fat from the depots or the food. The

behaviour of thiamine in causing an increase both in liver fat and in depot fat is in contrast to this general finding, and considered in conjunction with the third point that fat accumulates in the liver on low choline diets which are essentially fat-free, leads to the implication that fat is synthesised in the liver under the action of the vitamin, and that its accumulation is due in part to difficulties of transport. Hence if thiamine causes fat synthesis in the liver from pyruvic acid, its rôle in the production of the fatty liver would be explained, as would be the increase in depot fat; similarly, the greater need for thiamine in diets of low fat content would be accounted for by its being required for any synthesis of fat from carbohydrate, such a synthesis being made unnecessary by the provision of extra fat in the diet (67).

In a further paper McHenry & Gavin (68) report that thiamine causes an increase in the amount of fat, chiefly in the livers, of rats and pigeons fed on a fat-free diet high in carbohydrate. Lactoflavin was without action alone, but in conjunction with thiamine caused a further increase in liver fat. While choline reduced the liver fat it did not increase the depot fat, although vitamin B₆ was able to effect such an increase. The problem of the interaction of the many different factors that control liver-fat content grows increasingly more complex with successive years, and much further work will clearly be necessary before a more complete picture is obtained.

The problem of the essential fatty acids has been further studied. The acrodynia of rats on a fat-free diet was cured by small doses of ethyl linoleate, of peanut, wheat germ, corn, and wesson oils, and by larger doses of coconut oil and butter fat; hydrogenated coconut oil and the unsaponifiable fraction of wheat germ oil were without such activity, the whole of which was present in the fatty acid fraction from the oils, and could be concentrated by separation from acetone at -50 to -75°C . (69).

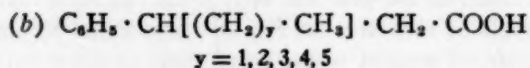
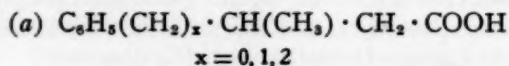
While the addition of methyl linoleate improved the growth rate of rats on a fat-free diet, ethereal extracts of brain and liver gave a further response, possibly on account of their arachidonic acid content (70, 71); methyl linolenate was without effect. On the other hand, McCollum *et al.* have used adequate diets of lower fat content than the so-called "fat-free" diets previously employed; they added methyl linoleate and a vitamin-E concentrate and obtained no evidence of the existence of any hitherto unknown fat-soluble substance necessary for growth and reproduction in the rat (72).

Oxidation.—Studies on the mechanism of the oxidation of fatty

acids are still chiefly concerned with the mode of formation of ketone bodies and the reasons for their accumulation.

The use of deuterium-substituted fatty acids by Morehouse (73) has given some interesting information on the question of the mechanism of β -oxidation. She fed $\alpha\beta$ - and $\beta\gamma$ -deuterobutyric acids to fasting rats which had received either a stock diet or a high-fat low-protein diet to induce endogenous ketonuria. About one-fifth of the β -hydroxybutyric acid excreted when $\beta\gamma$ -deuterobutyric acid was administered contained deuterium while only one twenty-fifth of the β -hydroxybutyric acid contained deuterium when $\alpha\beta$ -deuterobutyric acid was given. Control experiments showed that there was no exchange of deuterium with hydrogen in β -hydroxybutyric acid *in vivo* or *in vitro*. Besides showing once again that β -hydroxybutyric acid is in part, at any rate (cf. 75), produced from butyric acid, these results seem to provide further evidence for $\alpha\beta$ -desaturation.

The preliminary note of Carter *et al.* (74) is also of interest in this connection. They have studied the urinary excretion products in dogs after the administration of the sodium salts of ω -phenyl- β -alkyl fatty acids of two types:



None of the acids yielded appreciable amounts of benzoic or phenyl acetic acid in the urine; substances of type (a) did, however, yield varying amounts of unsaturated acid, and all the acids excreted were either in the free state or conjugated with glycine.

Apart from an extended use of deuterium or from experiments of the type of those just described, it is clear that little progress is likely to be made in the understanding of fat oxidation until the enzyme systems involved can be studied *in vitro*. Some progress in this direction is reported by Leloir & Muñoz (75). These workers have carried out extended experiments with liver slices and with a cell-free liver extract in order to obtain information of greater quantitative significance on the β -oxidation of fatty acids by the liver. By the use of the Warburg technique and a micro method for the estimation of the fatty acids by distillation and oxidation with dichromate, they

studied the rate of oxidation of, and formation of, acetoacetic and β -hydroxybutyric acids from normal fatty acids with from one to eight carbon atoms. Their paper should be consulted for fuller information; here it may be mentioned that the relative rates of disappearance of the acids were: formic, 1.5; acetic, 5.0; propionic, 2.0; butyric, 9.0; valeric, 2.1; hexanoic, 6.0; heptanoic, 3.0; and octanoic, 6.0. The acids containing an odd number of carbon atoms yielded only traces of ketone bodies, but the fact that 20 per cent of the acetic acid metabolised was converted to acetoacetic acid is of much interest, since it suggests that butyric and higher fatty acids may not be the sole fatty acid source of acetoacetic acid and related compounds; 80 to 90 per cent of the butyric acid which disappeared was accounted for by ketone body formation, the remainder probably being oxidised by some other route. The results with octanoic acid provide further evidence for the belief which is now being established that higher fatty acids can give rise to more than one molecule of ketone body. The yield of ketone body lay between that required by successive β -oxidations and that resulting from one molecule of octanoic acid giving rise to two molecules of ketonic acid. The fact that acetic acid was identified as one of the products of oxidation suggests that octanoic acid is oxidised by two methods, part giving rise to one molecule of ketonic acid and two of acetic acid, and the other part to two molecules of ketonic acid. Of great interest is the fact that, while previous workers have failed to obtain oxidation, *in vitro*, of fatty acids except by the use of liver slices, these authors found it possible to prepare a cell-free liver extract which would oxidise butyric acid. This preparation was found to be rapidly and irreversibly inactivated especially under anaerobic conditions; when thoroughly oxygenated, however, it oxidised butyric acid almost quantitatively to acetoacetic and β -hydroxybutyric acids. The rate of oxidation of butyric acid was increased by the addition of Szent-Györgyi's C_4 dicarboxylic acids, but without a proportionately large increase in the rate of ketone body formation; moreover a larger fraction of the acetoacetic acid was reduced to β -hydroxybutyric acid.

A paper by Bernheim & Bernheim (76) may be mentioned here in which they describe investigations on the oxidation of phospholipids by suspensions of rat or guinea-pig liver. The addition of certain vanadium compounds to such preparations at pH 6.7 increased the oxygen uptake. Moreover, a thoroughly washed liver suspension would rapidly oxidise added phospholipid in the presence of vanadium

salts. These authors showed further that salts of manganese, and to a lesser extent of cobalt, inhibited the oxidation of phospholipid by the washed liver protein-vanadium system; nickel, iron, titanium, and chromium had no effect (77).

With regard to the more general question of the reasons for the accumulation of ketone bodies, Mirsky *et al.* (78) point out that previous experiments, in which was measured only the excretion of ketone bodies, after injection of sodium β -hydroxybutyrate, may lead to wrong conclusions since they do not take into account any endogenous formation or utilisation. In order to avoid the complications introduced by urinary excretion, they used nephrectomised female rats and determined the acetone body content of the whole animal. They found that the utilisation of sodium β -hydroxybutyrate, injected into such animals is uninfluenced either by fasting or by glucose administration. These important results led them to the conclusion that the effect of carbohydrate in abolishing ketonuria is due to an antiketogenic rather than a ketolytic action.

The fact that fatty infiltration of the liver so often accompanies ketosis may also suggest that the action of carbohydrate and glycogenetic substances is antiketogenic. Thus Fraser *et al.* (79) have studied the particular susceptibility to ketosis, of multiple pregnant ewes, and find that, in contrast to barren ewes, pregnant ewes develop ketonaemia on a sub-maintenance diet. The ketonaemia can be removed by increasing the caloric intake irrespective of the nature of the diet. In the untreated cases which were sufficiently severe to cause death, post mortem examination showed in all cases multiple pregnancy and livers infiltrated with fat. It appears that in the absence of body stores of carbohydrate sufficiently adequate to meet the greater metabolic demands, an increased utilisation of fat causes the transport of extra fat to the liver for oxidation with a corresponding increase in ketone body formation. It is of interest, however, that MacKay *et al.* (80) have confirmed the fact that in rats, choline administered during fasting has no effect on the resulting ketosis, nor does the inclusion of choline in the diet previous to fasting, influence the subsequent fasting ketosis although fat accumulation in the liver is prevented. They have also studied the effects of varying the protein (caseinogen) content of a diet containing 30 per cent of fat on the subsequent fasting ketosis in rats, and found that the ketone body excretion is conditioned by the previous protein intake. An intake of 2.8 gm. of protein per day practically prevented the occurrence of ketonuria even

when fasting was continued for five days. This interesting finding calls for further study.

Fat deposition.—The problem of the deposition and utilisation of reserve fat in animals continues to receive chemical study, and a series of instructive experiments have been reported by Longenecker (81, 83, 84). Very convincing proof of the synthesis of fat from protein has, for instance, been provided. After a preliminary feeding period, rats were fasted until they had lost some 25 per cent of their body weight and were then refed on a diet containing 96 parts of caseinogen until they had regained their initial body weights. During the re-feeding, the carcass fat rose from 1.1 per cent to which it had fallen during fasting, to 10.5 per cent, and nearly one-third of the increase in body weight was due to fat synthesised from protein. Detailed analysis of the fatty acids of the synthesised fat showed a 50 per cent increase in the percentage of C_{16} acids as compared with the fat at the end of the preliminary feeding period, and it is not without interest that a fourfold increase occurred in the amount of hexadecenoic acid. This constituted 15.6 per cent of the total acids. Similar studies with a high carbohydrate diet following starvation gave a similar degree of fat synthesis and, as might be expected from theoretical considerations, there was little difference in the composition of the fat whether it was synthesized from carbohydrate or from protein (81). The synthesis of fat from protein by the rat has also been reported by Hoagland (82).

Longenecker has also studied the utilisation of the individual fatty acids in rats previously fattened on coconut oil until their body fat contained as much as 31.8 moles per cent of lauric acid; the animals were then fasted and their depot fats analysed after they had lost 15 and 30 per cent of their body weights (83). Similar methods have been applied in a study of the utilisation of depot fat resulting from the ingestion of diets which contained 84 per cent of corn oil; in this work the hardening effect of carbohydrate was also studied (84). These papers must be consulted for the detailed findings; suffice it to say here that the results of both of the two last studies give the impression that utilisation of the fatty acids deposited in the depot fat is probably not a specific utilisation of individual members, but a general utilisation of all the members. Hilditch *et al.* (85) have reported results which show that restricted feeding on a low fat diet causes the deposition of a softer fat in pigs than a liberal diet of the same constituents; more oleic and possibly hexadecenoic acids occurred while

linoleic and the higher unsaturated acids were derived from the dietary fat only.

Keil *et al.* (86) have carried out an unusual study in which they converted the mixed fatty acids of coconut oil to acids with one more carbon atom from which they then prepared triglycerides. Parallel experiments with coconut oil and the synthetic "coconut oil" containing C_{2n-1} fatty acids showed that the increase in the chain length of the fatty acids had essentially no effect on the iodine number of the fat deposited by animals receiving it, nor on its ease of hydrolysis by pancreatic lipase nor on the respiratory quotients of the animals.

Certain natural fats contain polyethylenic acids which show absorption in the ultraviolet region either before ("absorptive" acids) or after boiling with alcoholic potassium hydroxide ("pro-absorptive" acids); the problem of the occurrence in animal fat of acids showing ultraviolet absorption has been reported upon in a series of papers (87, 88, 89). Evidence is presented which shows that the intensity of the ultraviolet absorption of these acids is due to their content of certain polyethylenic compounds (87, 89). In the acids of butter fat, absorption occurs at 230 $m\mu$ and administration of cod liver oil to cows increases its intensity; however, the acids in cod liver oil which show "pro-absorption" at 270 $m\mu$ are not transferred to the milk fat; when tung oil which itself shows absorption at 270 $m\mu$ is given to cows the butter acids show the absorption at 270 $m\mu$ (87). In contrast, the hen apparently converts this latter acid to one which absorbs at 230 $m\mu$. Further evidence concerning the differences in the behaviour of cows, hens, and rats in their treatment of polyethylenic acids, based on absorption in the ultraviolet is presented (88). Catalytic reduction of tung oil causes a shift in the absorption maximum from 270 to 230 $m\mu$, as in the hen, while the change caused by refluxing "pro-absorptive" acids with alcoholic potassium hydroxide is a result of reorientation of the double bonds to conjugated positions (89). These studies are of interest in throwing light on the changes which tissues can effect in unsaturated acids without disruption of the molecules, and ultimately they may provide evidence of value on the question of desaturation.

Blood.—Thyroidectomy had no effect on blood cholesterol content in rats (90), but thyroid administration lowered the concentration of serum cholesterol in man (91); of fifty-nine cases of toxic goitre examined forty-nine showed hypolipemia (92). Thyroxine administration to cows caused hypolipemia which affected all of the plasma lipid

constituents, and increased by 50 to 60 per cent the secretion of milk fat with no appreciable change in its composition (93). Here also may be mentioned further work on the action of the anterior pituitary on lactation. Since prolactin-C gave a considerable increase in the yield both of milk and of milk fat in cows, while thyrotropic-C gave some increase in milk yield without an increase in milk fat, Folley & Young conclude that the action of anterior pituitary extracts on cows cannot be ascribed to a single substance (94).

Chaikoff *et al.* have continued their studies on the pancreatic factor involved in blood lipid control. Ligation of the pancreatic ducts in dogs caused a decreased concentration of all of the blood lipids, irrespective of body weight loss, and gave the same blood fat picture as that of depancreatized dogs maintained with insulin. The giving of pancreatic juice or raw pancreas to the latter animals caused a considerable rise in the blood lipid content (95); raw pancreas, if given immediately after operation, would prevent the fall in the blood lipid content of duct-ligated dogs, and if given later, would cause a rapid increase from the low level already reached. It is concluded that the active pancreatic substance is contained in pancreatic juice (96). Raw or desiccated pancreas was found to cause an increased concentration of plasma cholesterol esters and of phospholipids in children showing a low level of blood lipids consequent on infection (97).

In a further study of the action of anterior pituitary extracts on fat metabolism, Houchin & Turner have found that anterior pituitary extracts depressed the plasma lipid content of rabbits by an average of 36 per cent after eight hours; there then followed a return to normal after twenty-four hours. The active substance was not associated with fractions rich in the lactogenic, thyrotrophic, gonadotrophic, or carbohydrate hormones, and the effects observed were not obtained with thyroxine or extracts of adrenal cortex (98). In a further paper, the authors record similar observations on the blood lipids of the guinea pig, and find that the extracts also increase the amount of fat in the liver; they propose the use of the depression in the blood lipid content of the guinea pig as a method of assay (99).

An action of estrogens in elevating the blood lipid content has been reported. Cocks receiving estradiol benzoate for periods of three to twelve weeks developed an astonishingly intense lipaemia. The highest figures in the control birds were: Lipoid phosphorus, 10.4 mg. per cent; total cholesterol, 128 mg.; free cholesterol, 33 mg.; and total fatty acids 10.6 m.eq. per cent; in the injected birds the corre-

sponding figures were 201, 1059, 790, and 573. From their pale appearance, the livers were probably fatty (100). Similar findings are reported by Zondek & Marx (101) who point out that the spontaneous lipaemia which occurs in hens during the laying season does not occur in the cock save under the action of estrogenic hormones. They also report an intense activity of diethylstilbestrol, 24 mg. of which, given over six days, increased the blood fat from 125 to 5430 mg. per cent, a forty-fold increase; the lipaemia of the normal laying hen represents no more than a five-fold increase in blood fat.

Exposure of cats and dogs to atmospheres containing 10 per cent of oxygen for periods of up to six hours had no effect on the blood lipids either during fasting or active fat absorption; a transient decrease, however, was observed in the rabbit (102).

Studies have been reported on alimentary lipaemia in clinical diabetes (103, 104, 105). No correlation was found between the level of the lipaemia, its duration, the degree of liver damage as shown by a liver function test (103), and the diabetic symptoms (104), although alimentary lipaemia was found to run parallel with the degree of disorder of carbohydrate metabolism (105); administration of betaine chloride improved the liver function but did not affect the blood lipid content (103). In contrast to the diabetic, insulin shock treatment of schizophrenic subjects caused an immediate and prolonged rise in the serum lipid content (106). Studies of the total and phospholipid fatty acids of the serum of fourteen normal (107) and seven eczematous infants (108) have been reported. In the latter, both fatty acid fractions were less unsaturated than in the normal, in which the phospholipid fatty acids had a higher molecular weight, 303, than the total fatty acids, 288. A case of idiopathic familial lipaemia, characterised by a neutral fat hyperlipaemia and hepatomegaly was reported; in the acute stages, there was a sudden reduction of the blood lipid content with enlargement of the liver; neither thyroxine, insulin, lecithin, choline, nor anterior pituitary preparations had any effect, but alleviation followed the use of a low fat diet (109). In a case of idiopathic steatorrhea, a low fat diet was found of value, and there was no evidence for a high fat excretion into the intestine; reduction of calcium intake facilitated absorption (110). Blood lipids in cancer patients were not found to be characteristic of the disease (111).

Cholesterol.—Studies *in vitro* have been reported concerning the enzymes in blood and tissues which cause the synthesis or hydrolysis of cholesterol esters (112). Cholesterol feeding of guinea pigs caused

severe anaemia and the spleen became ten times its normal size within nine weeks; in this work cholesterol deposition was observed in the liver, spleen, heart, lungs, and blood (113). Cholesterol feeding experiments on the rat, rabbit, and guinea pig with reference to cholesterol atheroma were also reported (114). In experiments on the production of tumours in rats by ultraviolet irradiation of up to 5,000 hours spread over 259 days, irradiation caused an increase in the cholesterol of the skin (115); similar results were obtained on rats but not on guinea pigs or mice (116). In rabbits suffering from Anjeszky's disease, the blood cholesterol increased significantly (117); rabbits with experimental herpes virus infection showed no change in blood cholesterol either before or after treatment with thiamin or sulfanilamide (118). Thyroid administration did not accelerate the removal of intracutaneous cholesterol deposits in rabbits (119).

Methods.—Proposals have been made for overcoming the well known difficulties encountered in the determination of cholesterol by the Liebermann-Burchard reaction (120) and for the use of the Tschugaeff reaction for cholesterol estimation (121). Kelsey has reported that under suitable conditions the lipase of castor oil beans and an extract of pancreas made with dilute ammonia will hydrolyse triglycerides without affecting cholesterol esters (122, 123). Such a method would overcome previous difficulties in making it possible to analyse the fatty acids linked to cholesterol, a matter of importance in the study of the participation of cholesterol in fat metabolism. Application of the method to the lipids of dog plasma suggested the presence of an unknown ester resistant to hydrolysis (124). A further proposal for following fat metabolism by use of a labelled fat is that involving application of the findings previously discussed (see p. 244) (87, 88, 89) to the linoleic acid of corn oil (125); this method may prove of very great general value, but in its use the results discussed earlier may need consideration.

Thannhauser *et al.* have modified their original method for the estimation of lecithin, cephalin, and sphingomyelin in body fluids and tissues, and record figures for the amounts of these phospholipids in human blood serum, brain, lung, spleen, kidney, liver, and heart (126, 127). A simple procedure for the study of fat absorption has been proposed (128).

The difficulty of preparing blood phospholipids free from extraneous nitrogen compounds is well known. The nature of these contaminants has been investigated, with the finding that many substances

which are insoluble in light petroleum are soluble in light petroleum solutions of the phospholipids; the chief contaminant was found to be urea (129, 130).

Miscellaneous.—The chemical composition of a number of tissues and fluids has been determined. Thus, human blood platelets contain 16 per cent of their dry weight as lipids, made up of phospholipids, 12; free cholesterol, 2; cholesterol esters, 1; and glycerides, 1 per cent; two-thirds of the phospholipid is kephalin (131). The cell nuclei of certain tissues contain considerable amounts of lipids, especially cholesterol and phospholipids which are probably sphingomyelins, and lecithins and kephalins of low iodine number (132). The phospholipids of human, pig, and ox bile have also been studied (133). Diets which contain much fat decrease the excretion of uric acid, an effect not due to the consequent ketosis; diets high in carbohydrate and of equal calorific value have no such effect. The results indicate the use of the latter diets in the treatment of gout (134). In confirmation of previous work, the lipids of the spleen in a case of Niemann-Pick disease were analysed; on hydrolysis of the sphingomyelin, sphingosine, lignoceric acid, palmitic, and stearic acids were isolated; 70 per cent of the monoamino phospholipids was kephalin (135).

Two reviews have been published: *Fat Transport in the Animal Body* by Bloor (136) and *Fat Metabolism in the Animal Body* by Smith (137).

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CARBOHYDRATE METABOLISM

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DIGESTION AND ABSORPTION

The lack of glucose absorption in the stomach has been generally accepted in the recent literature as a well-established fact. The earlier results are now challenged by clinical observations with a multilumen stomach and duodenal tube (154) and by experiments on the anesthetized dog with a clamp on the pylorus (136). In the latter experiments, one hour after the introduction of about 90 gm. of glucose in 40 to 47 per cent concentration, from 7 to 23 gm. could not be recovered from the stomach and various degrees of hyperglycemia were found. Recovery was complete within experimental error when concentrations of glucose below 7 per cent were used. The authors conclude that gastric absorption takes place from glucose solutions of high concentration. Various degrees of glycolytic activity in the pylorus could be stimulated by the ingestion of different foods (170, 174).

The rate of gastric emptying of cereal starch and consequent intestinal absorption of sugar was in proportion to the size of the meal in fasted rats, although hydrolysis of the starch exceeded absorption in the early stages (67). In measuring the digestion of raw starch by recovery in the feces, factors to be considered are freezing the meal before ingestion and bacterial fermentation in the intestinal tract (16).

Oral administration of the rare sugars, melezitose, turanose, and trehalose led to an increase in liver glycogen in the fasted rat, whereas the results with raffinose and melibiose were negative. The data are interpreted as indicating the presence or absence in the alimentary tract of the corresponding enzymes (36). Contrary to earlier reports on other species, Davidson & Garry found that only minute amounts of hexoses or xylose were absorbed in ninety minutes from the large intestine of the anesthetized rat (49).

Several investigators have studied the influence of the endocrines on intestinal absorption. Removal of the rat's pituitary gland decreased the intestinal absorption of glucose by about 30 per cent if compared to litter mate or weight controls, less if compared to senile

controls (144). Blood sugar changes were not significantly different in the four groups, hence did not indicate the degree of intestinal absorption. The increase in muscle and liver glycogen in the hypophysectomized rats after the glucose meal was only about 50 per cent of that found in the controls.

Verzár's theory of the influence of the pituitary gland and the adrenal cortex on selective absorption of glucose and galactose over fructose and xylose from the small intestine has been supported by Minibeck (133). After frogs were hypophysectomized or adrenalectomized, the selective absorption disappeared since the rate for the four sugars was about equal. Additional evidence is accumulating to show that sodium salts restore the normal glucose absorption rate in the absence of the adrenals. Althausen, Anderson & Stockholm (2) have confirmed the work of Deuel *et al.* (51) with sodium chloride. Sodium lactate or pyruvate (105) was also effective in adrenalectomized rats. Laszt (106) has reported that the inhibiting effect of iodoacetic acid on glucose absorption could be nullified by a previous injection of sodium chloride or carbonate.

Lundsgaard (117) has found an increased content of esterified phosphate in intestinal mucosa during absorption of both glucose and fructose. The higher values obtained in the case of fructose were interpreted as being due to slower dephosphorylation, thus accounting for the well-known slower absorption of this sugar. Another phase of active preferential absorption, the movement of glucose against a concentration gradient, was studied by Bárány & Sperber (12). The concentration of glucose in the lumen of the intestine continued to fall after it had passed below the blood sugar level of about 190 mg. per cent in the anesthetized rabbits. Sodium sulfate or sorbose were used as controls in adjacent segments to indicate possible fluid changes.

From experiments on rats after removal of various endocrine glands, Althausen (1) has proposed a galactose tolerance test for intestinal absorption in clinical endocrine disorders. The excretion in the urine of 20 to 30 per cent of the dietary galactose by rats on a skim milk diet can be prevented by even chain fatty acids of twelve or more carbon atoms, according to Schantz & Krewson (151).

BLOOD SUGAR AND INSULIN

The action of injected insulin and glucose in gradually restoring the normal rate of carbohydrate oxidation after fasting or fat feeding has suggested that the impaired metabolism of sugar in these

conditions is related to a reduced production of insulin by the pancreas and a diminished sensitivity of the tissues. Best, Haist & Ridout (18) have presented evidence indicating a lower rate of insulin production in experiments on the insulin content of the rat's pancreas. Fasting to a weight loss of over 20 per cent reduced the insulin content by 50 per cent or more. With subsequent fat feeding for six days, the insulin content continued to fall, whereas ingested sugar led to a partial recovery, and a balanced diet to a complete recovery by the sixth day.

Insulin sensitivity has been studied in many ways. When it is judged solely on the basis of blood sugar changes, it should be remembered that these may also reflect the influence of other hormones and reactions in the body. The carbohydrate content of the diet had little effect on the difference in area between the glucose tolerance and the glucose plus insulin blood sugar curves when determined for a period of one hour with a constant dose of insulin (90). The apparent discrepancy with earlier work is explained as due to the nullifying effect of alimentary hyperglycemia, which is calculated from glucose tolerance experiments with varying doses of insulin. The relationship of these results to the earlier work on insulin sensitivity is discussed by Himsworth (89) in lectures on blood sugar control and diabetes. Contrary results are reported by Courtice, Douglas & Priestley (44) since no reduction in insulin sensitivity was found after a low carbohydrate diet or one and one-half hours after finishing prolonged moderate exercise. In both conditions, however, there was a low basal respiratory quotient and the typical reduced tolerance for glucose. With the low carbohydrate diet a delayed rise in respiratory quotient after glucose ingestion was evident.

Sympathectomy in the dog increased the sensitivity to insulin without interfering with the normal blood sugar regulation in response to epinephrine, exercise, or the glucose tolerance test (28). Stimulation of the cervical sympathetic nerve or its superior ganglion produced no significant change in blood sugar in either acute or chronic experiments (87), thus contradicting the work of earlier investigators. Stellate ganglionectomy in the dog did not influence the insulin sensitivity, the glucose tolerance, or the insulin convulsions (42). Section of the hypophyseal stalk in the monkey did not change the reaction to insulin except in one animal with extensive degeneration in the anterior lobe of the pituitary (27). Various degrees of recovery from the hypersensitivity characteristic of removal of the pituitary

gland was found in dogs between the ninth and sixteenth postoperative months (20). Soskin, Levine & Heller (159) have shown that the insulin sensitivity of the hypophysectomized dog is not affected by the daily administration of thyroxin.

For a quantitative measure of insulin action Drury & Greeley (56) have balanced the intravenous infusion of insulin and glucose in the depancreatized dog so that a normal level of blood sugar is maintained. Only 0.005 to 0.035 of a unit per kg. per hour is required for the "basal" action of preventing excessive protein catabolism in the fasting condition. When a larger amount is given, another action is seen, that of removing glucose from the blood for oxidation and storage. In other experiments (76) the oral administration of more sucrose than glucose was necessary to balance a constant amount of insulin. A fall in blood sugar followed the ingestion of 10 gm. of sucrose by a diabetic animal in glucose-insulin equilibrium.

If on successive days insulin is given until convulsions occur, the glucose tolerance of the dog is not changed although the resistance to convulsions is lowered (43). After insulin was diverted around the liver by venous anastomosis the reduction in galactose tolerance after four days of fast was not significantly altered (132).

The urinary secretion of a blood sugar raising principle by depancreatized dogs was not affected by the administration of insulin and raw pancreas but it failed to appear in hypophysectomized animals (37).

DEPOSITION AND TRANSFORMATION

The glycogen concentration in the liver of rats eight to eleven days old is one-third that of older rats although the muscle glycogen is approximately the same. During the first six weeks of postnatal life, liver glycogen accumulates to a value slightly above that of normal adults (86).

An altered response to ingested carbohydrate and changes in base excretion occurred as soon as twenty-four hours after adrenalectomy in rats (3). In comparing the amount of ingested glucose stored to that oxidized, Russell (146) has found that anterior pituitary extracts administered to adrenalectomized rats lowered liver glycogen but produced no effect on carbohydrate oxidation. When cortical extract was given, muscle glycogen was raised and oxidation of carbohydrate lowered. When both were injected, the effects on lowering oxidation and increasing storage were greatly enhanced. Further studies on the

ability of cortical extract to maintain normal carbohydrate stores in the absence of the pituitary have been reported by Corey & Britton (38), and by FitzGerald & Verzá (63). This work supports the hypothesis that the pituitary exerts its carbohydrate effects largely through the adrenal cortex. Britton (26) has further suggested that insulin influences glycogen storage through the adrenal cortex, in view of evidence that, in the adrenalectomized animal, glucose and insulin are unable to give rise to glycogen deposition.

Brentano (23) has studied the alleviation by insulin of impaired glycogen formation from administered sugar, using rabbits with creatinurias caused by starvation and phlorhizin. Insulin caused storage of glycogen in the muscle of these animals, considerably at the expense of liver glycogen deposition. Animals treated with thyroxin were not helped by insulin (23, 110). Loubatières, Monnier, and Cristol have shown graphically the fall in liver glycogen and rise in liver fat resulting from pancreatectomy (114) and the reversal of both changes to normal by insulin treatment (47). Gemmill noted a glycogenic effect of insulin on rat diaphragm *in vitro* (69). Glycogen is deposited in the rat liver with the same amount of water, potassium, and acid-soluble phosphate as is found associated with the other solids of the liver (62).

Lactate infused into rabbits with diphtheritic toxemia was converted to glucose more slowly than normally, and liver glycogen formation was very low. These defects were remedied somewhat by the previous injection of adrenal cortical extract (50). Propylene glycol, in contrast to ethylene, diethylene, and dipropylene glycols, produced muscle as well as liver glycogen in the rat (82). Todd, Myers & West (169) reported that sorbitol was glucogenic in dogs and glycogenic in rats, and that mannitol could give rise to glycogen in rats under certain conditions. *d*-Sorbitol, styracitol, and *l*-sorbitol fed in a fat diet to rats all caused increases in liver glycogen (30). *l*-Xylulose, and possibly *d*-xylulose, was glucogenic in the depancreatized dog (104). Glucosamine yielded extra sugar in phlorhizinized rabbits, and elevated the blood sugar in normal and splanchnectomized rabbits (173). The intravenous injection of pyruvate into normal animals has been found to produce a rise in blood sugar and lactate (116).

The effect of a high protein diet on increasing glyconeogenesis in the liver was not evident after thyroidectomy. A previous high fat diet accelerated the accumulation of liver glycogen after depletion with phlorhizin. The increased nitrogen excretion indicated protein

as the principal source of the carbohydrate (163). The daily administration of thyroxin to fasting hypophysectomized dogs raised their endogenous protein catabolism to the normal level and thereby maintained a normal blood sugar concentration (159). Sternheimer (164) has linked protein synthesis in the liver with the depleted glycogen resulting from thyroxin administration. Adrenal cortical extract also is effective in accelerating glucose formation from protein (99).

Phlorhizinized dogs with ligated pancreatic ducts had fasting D:N ratios similar to the Lusk ratio of 3.6 in the unoperated phlorhizinized animals. When casein was ingested, the ratio was similar to Minkowski's 2.8 in the depancreatized dogs. Duct ligation lowered the yield of glucose from ingested casein in the phlorhizinized group (75).

Longenecker (112) has found an increase of 15 per cent in the amount of C_{16} fatty acids in rat depot fat, when dietary carbohydrate or protein is the source instead of fat. Further evidence has been presented to substantiate the role of thiamin in fatty acid production from carbohydrate (127).

MOBILIZATION

The glucose-lactic acid cycle involving muscle and liver, worked out earlier under conditions of hyperglycemia and hyperlactacidemia, probably does not play a significant role at normal or lowered blood values. By use of the angiostomy technique, Cherry & Crandall (33) have been able to show that, in normal unanesthetized dogs, leg muscle retained 4.8 mg. of glucose per 100 cc. of blood, and liberated 3.1 mg. of lactate. The liver liberated 9.1 mg. per cent glucose, but showed no significant lactate changes. The authors considered that the heart and brain removed and oxidized directly the lactic acid produced under resting conditions. Himwich, Fazekas & Nesin (93) have reported similar findings. The first authors (46) have correlated hepatic glucose output and arterial blood sugar level: in the group of dogs with lower blood sugar (average 74 mg. per cent), the output was 6.2 mg. per cent, while in those with an average level of 83 mg. per cent, the output was 22.5 mg. per cent. Twenty minutes after injection of insulin, the hepatic glucose output increased in the first group, but fell in the second, so that hepatic glycogenolysis could be increased or decreased by insulin. Part of this may be the mechanism by which insulin causes deposition of muscle glycogen at the expense of the liver stores. The authors consider that changes in blood flow would not affect the interpretation of their results. The hepatic

output of glucose was found below normal in hypophysectomized, adrenal-denervated, or adrenalectomized dogs, although an essentially normal formation of glucose from glycine suggests that gluconeogenesis from amino acids is not at fault. These operated animals all responded to insulin with a fall in glucose output, supporting an endocrine explanation of the carbohydrate defects.

Amelioration of diabetes mellitus is described by Bloomfield in a patient developing Addison's disease (19). Adrenal cortical extract was effective in raising the blood sugar level but a marked hypoglycemia followed treatment with desoxycorticosterone acetate in daily doses up to 25 mg. Harrison & Harrison (84) reported that adrenalectomized rats fasted forty-eight hours require 2.5 mg. per day of synthetic desoxycorticosterone to keep the blood sugar at normal levels, whereas 1.25 mg. suffices to keep the serum sodium, potassium, and nonprotein nitrogen normal.

Selye & Dosne (153) have found that cortical extract decreases the extent of both the insulin and adrenal changes in blood sugar. Changes in blood sugar following estradiol or progesterone injection were studied by Zunz and La Barre in relation to adrenal activity (175).

Brentano and co-workers have noted the absence of increased blood lactate after adrenalin in animals with a creatinuria (88, 126). When glucose was administered to rats, followed in three hours by adrenalin, the normal animals showed a fall in liver and muscle glycogen, combined with marked rises in blood sugar and lactate. In animals phlorhizinized to produce a creatinuria, there was no change in liver glycogen or in blood lactate, muscle glycogen fell, and blood sugar rose. The fall in muscle glycogen, although as extensive as in the controls, did not produce any rise in blood lactate, evidence which Brentano interpreted as suggesting hyperactive glycogenesis.

OXIDATION IN THE WHOLE ORGANISM

Careful studies of men performing strenuous exercise furnish additional evidence of the relationship between carbohydrate and fat oxidation in the normal intact body. Christensen & Hansen (34) employed continuous exercise (ten times basal) at a constant rate and found the steady state was reached in ten to fifteen minutes, while Margaria (123) used thirty-minute cycles of light and heavy work. Their results are in general agreement. When the basal respiratory quotients were high, the quotients during work fell gradually to about 0.83.

In the light work periods the quotients were lower (123). After a low carbohydrate diet, the exercise quotients were the same as or slightly above the basal quotients of 0.71 to 0.76; thus, more than 90 per cent of the energy came from fat (34). The intensity of the work was a factor determining the proportion of carbohydrate to fat oxidation as well as the marked reduction in carbohydrate stores, which may amount to between 200 and 400 gm.

Under basal conditions, respiratory quotients below about 0.78 were associated with an increase of 8 to 15 per cent in oxygen consumption, in agreement with the older literature which has been quoted as evidence for carbohydrate formation from fat. A more plausible explanation may be that the inefficiency of fat when the quotients are low is due to the loss of oxygen from the body in the form of excreted ketones derived from fat. The data presented on ketone excretion are inadequate to decide the question. In the exercise experiments, the decline in muscular efficiency with a fall in respiratory quotient is denied by Margaria and not well supported by the data of Christensen & Hansen, since at quotients of about 0.92 and 0.79, the net energy differences were approximately -3, +5, and +2 per cent. Although the general trend of the blood sugar level was downward with the fall in carbohydrate oxidation, individual experiments showed no relationship between the two. Administration of glucose elevated the blood sugar without appreciably changing the amount of glucose oxidized. Similar blood sugar changes have been found in exercising dogs (149).

The effect of epinephrine during milder exercise has been observed by Courtice, Douglas & Priestley (45). Epinephrine hyperglycemia was less during exercise than during rest while the blood lactate changes were about the same in both conditions. As in the resting state (44) the changes in respiratory quotient seemed to correlate with the blood lactate changes rather than to indicate an increase in carbohydrate oxidation, despite the hyperglycemia.

The relative amounts of ingested carbohydrate which are oxidized and stored by the human subject under different conditions have been studied by Newburgh and co-workers (97, 139). Many markedly obese middle-aged patients with hyperglycemia and glycosuria had a normal glucose tolerance when their weight was reduced to normal but suffered a recurrence of defective carbohydrate metabolism with a return of the obesity (139). Since the respiratory data show a normal oxidation of carbohydrate when the subjects were obese, the in-

tolerance is explained as a deficiency in glycogen deposition, probably related to a fatty liver rather than a decreased insulin secretion. This defect in carbohydrate metabolism was not found by Watson (172) in younger individuals with a mild obesity. In diabetic patients under insulin treatment, Bridge & Winter (25) reported normal respiratory quotients (ten-minute periods) associated with wide fluctuations in blood sugar concentration.

In undernutrition, impaired oxidation and preferential storage of carbohydrate have been demonstrated by observing the total respiratory exchange in human subjects for several days of controlled diet (97). On a low calorie, low carbohydrate diet the amount of carbohydrate oxidized may exceed that ingested. If a more drastic deficit in stored carbohydrate, equivalent to 100 to 200 gm., was caused by one or two days of fast, a considerable part of the ingested starch and sugar was retained rather than oxidized.

The impaired oxidation of glucose produced by a short fast can be improved by prolonging the fast to a weight loss of 40 to 50 per cent (31). As in the earlier report (13) on the depancreatized dog, the carbohydrate changes were found associated with changes in creatine-creatinine excretion, but not necessarily with a premortal increase in protein catabolism.

The production of a persistent diabetes in dogs by the prolonged administration of anterior pituitary extracts, as described by Young, has been confirmed in several laboratories (11, 29, 53, 85). The persistent diabetes does not develop in all of the treated animals (53) even after partial pancreatectomy (11). The carbohydrate metabolism is similar to that of partially depancreatized dogs according to Dohan & Lukens (53) who found in some animals low protein metabolism with little glycosuria on fasting and low D:N ratios on a meat diet. Similar results were obtained by Loubatières (113), as well as basal respiratory quotients of about 0.75 with no diabetic elevation of total metabolism (85). The hyperglycemia of a temporary diabetes during seven days of subcutaneous injection of extract was accompanied by a fall in the insulin content of the pancreas to about one-fifteenth of the normal value, then recovery occurred in a few days after withdrawal of extract (17). In two instances of permanent diabetes, the pancreatic insulin was too small to estimate.

Besides histological evidence of damage to the islet tissue (53), a stimulating effect on the adrenal cortex, thyroid, and other tissues has been noted in the early stages by Ham & Haist (81). In contrast

to the diabetogenic effect when the pancreas is present, Dohan & Lukens (54) report that prolonged administration of anterior pituitary extract in the absence of the pancreas produced an amelioration of diabetes resembling that seen in the Houssay preparation. These studies of pituitary diabetes suggest that the variable results may be explained as a balance between the effects on the pancreas and on other tissues such as the adrenal cortex, since carbohydrate oxidation in the adrenalectomized depancreatized dog is similar to that in the Houssay animal (32). A complementary action between anterior pituitary extract and adrenal cortical extract in the absence of the adrenal is proposed by Russell (146). The two extracts together suppressed the relative oxidation of ingested glucose considerably more than did cortical extract alone. Pituitary extract alone was without effect on the amount oxidized.

Harned & Cole (83) have made a detailed presentation of the differences between the Wistar and the Yale strains of albino rats, each raised for three generations on the same diet and under the same conditions. The Yale rats showed changes characteristic of hyperactivity of the anterior pituitary, such as higher fasting blood sugars, lower glucose tolerance, and insulin resistance. Orten & Devlin (141) also found a low glucose tolerance in 40 per cent of the adult Yale rats, as compared to the Wistar strain and fifty-day-old Yale rats. The low tolerance was restored to normal when sodium chloride was administered together with the sugar. This was also true in a group of partially depancreatized animals.

The defective carbohydrate oxidation first noted in terms of altered pyruvate metabolism in tissue studies on B₁-avitaminotic pigeons has stimulated considerable work on this important intermediary. Banerji & Harris (7) propose a quantitative estimation of the severity of the deficiency by the urinary excretion of bisulfite-binding substances, but pyruvic acid determinations are more reliable. Lu (115, 145) and Bollman & Flock (21) confirmed the elevated blood and tissue pyruvate levels in beriberi and experimental vitamin-B₁ deficiency. As soon as five hours after the administration of 5 to 10 mg. of thiamin the blood pyruvate showed a remarkable fall (145). The specificity of the relationship is challenged by von Euler's observation of an equally increased blood pyruvate in A-avitaminosis in rats (59).

Conditions which alter carbohydrate oxidation have a parallel effect on the oxidation of alcohol (55). In cases of human alcoholism, the disappearance of alcohol from the blood was found to be increased

by the simultaneous administration of glucose and insulin. Insulin alone was ineffective, and glucose alone was effective only when the blood alcohol content was very high (72). In contrast is the report of Mirsky & Nelson (135), that the liver is the principal factor responsible for alcohol utilization and that insulin is not essential for this process. Studies on various animal preparations as well as on excised liver tissue confirm the importance of the liver, but indicate the necessity of insulin in alcohol oxidation (35).

OXIDATION IN ORGANS

Brain.—The electrical activity of cerebral cortex, depressed by hypoglycemia resulting from hepatectomy, was restored to normal by the injection of glucose, mannose, and maltose. However, fructose, galactose, hexose diphosphate, glyceric aldehyde, succinate, fumarate, glutamate, lactate, and pyruvate (122, 137), all of which are metabolized by isolated brain tissue, failed to restore the rhythm. Himwich & Hoagland have continued their study of cerebral activity during induced hypoglycemia in schizophrenic patients (94). Measures which interfere either with the carbohydrate supply to the brain (insulin) or with its oxygen supply (metrazol, nitrogen inhalation, cyanide) have amply substantiated the concept of exclusive carbohydrate oxidation of this organ (61, 92). Himwich, Baker & Fazekas (91) have found whole infant rat brain to oxidize carbohydrate exclusively, similar to adult brain cortex.

Heart.—Subjecting rats to breathing pure nitrogen caused a marked fall during the first two minutes in heart glycogen, phosphocreatine, and adenylypyrophosphate. When the animals exercised in a treadmill, the cardiac content of all three compounds fell about one-third (152).

Evans (60) has summarized the recent developments in the study of the isolated heart, work which involves especially the substitution in the heart-lung apparatus of a mechanical oxygenator for the lungs, in order to eliminate the highly active blood sugar breakdown occurring in this organ. About 45 per cent of the energy requirement of the heart doing average work is furnished by lactate oxidation, and about 15 per cent by glucose oxidation. Contrary to expectation, the evidence indicates that lactate can be directly oxidized, but cannot be converted into glycogen, even when the reserve is low; only glucose forms glycogen readily. There probably is a pathway of carbohydrate oxidation not involving lactate, since isolated heart tissue can oxidize glucose in

iodoacetic acid poisoning (14). Pyruvate can be used as well as lactate, but its oxidation appears to follow a different path from that of glucose or lactate (22). The 40 per cent of unaccounted-for energy probably is furnished by fat oxidation, but chemical demonstration of this is not conclusive, due to the variable fat content of hearts, and to the high caloric value of fat as a foodstuff (48, 64).

Muscle.—Flock, Ingle & Bollman (65) have observed that direct stimulation of rat muscle with blood supply intact produced a rapid decrease in glycogen and phosphocreatine, with a slower fall in adenosinetriphosphate, and increases in lactate, inorganic phosphate and hexosemonophosphate. The peak of these changes was reached during the first minute of work. As the period of activity was continued, only lactate and hexosemonophosphate fell, the former, at least, probably by diffusion into the blood. In addition, the glycogen, phosphocreatine, and adenylypyrophosphate values remained low, only the latter rising toward normal after twenty minutes of stimulation. These changes cast some doubt on the significance of the well-known series of anaerobic reactions established, for the most part, with muscle and yeast extract systems, in the maintenance of continued muscular contraction (cf. 147). It should be pointed out, however, that the values obtained are dynamic rather than static, in that they represent breakdown plus resynthesis. During the early moments of activity, anaerobic breakdown to produce energy may be preponderant. As the supply of oxygen becomes adequate, resynthesis arrests further breakdown, but the high energy requirement during continued activity may prevent the return to resting levels.

Bollman & Flock (21), using the same rat muscle preparation, have found a rapid rise in pyruvate content during the first minute of stimulation. The excess pyruvate then disappeared, until resting values were obtained in two to five minutes, whether stimulation was continued or stopped. In vitamin-B₁ deficiency, the pyruvate levels started higher, but increased to about the same range as in normal animals, then fell off more slowly.

The perfusion of dog hind limbs resulted in loss of glucose from the perfusate if the level was higher than in the tissue. Correspondingly, the tissue gave up glucose to the fluid when the blood sugar level was low. The same general relationships were found in the depancreatized dog at higher levels (5). Genes reported increased sugar retention coupled with increased lactate production by hind-limb tissues of dogs under ether narcosis (70).

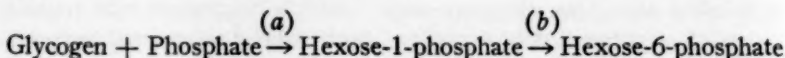
Lundsgaard and his co-workers have reported extensive work on the removal of glucose from cat hind-limb preparations. Since only about 10 per cent as much free sugar was found in the muscle as in the blood at varied levels, it was concluded that this is all extracellular. Therefore, the transfer of glucose from blood to muscle cells cannot be a simple diffusion, but must be an "active" process (as for instance, phosphorylation). The primary action of insulin is considered to be on the active process, with the other effects secondary (118). In the normal animal, the glucose uptake increased linearly with the blood sugar concentration from 200 to 600 mg. per cent, and the addition of insulin about doubled the rate of removal (120). In contrast, the uptake of glucose by isolated rabbit liver was unaffected by insulin (119). In phlorhizinized cats, glucose removal by muscle was normal (108). With previously depancreatized animals (120), the data indicated a normal uptake until about 350 mg. per cent, at which point it levelled off. This disagrees with Soskin's conclusion that muscle tissue of eviscerated depancreatized dogs utilized subnormal amounts of glucose until a blood sugar value of about 350 mg. per cent was reached, and paralleled the normal increase from 350 to 800 mg. per cent (158).

These experiments cannot be interpreted in terms of actual oxidation of the glucose removed, since no evidence (except for lactate) was presented concerning the fate of the sugar in the muscle. At the same time, caution may be expressed about reports concerning carbohydrate utilization (often interpreted as oxidation) in hepatectomized animals based on tissue carbohydrate analyses performed before and after the injection of glucose (57, 158, 160). A principal objection to these is the practical impossibility of judging the glycogen (or other carbohydrate) content of the entire body musculature from one or two isolated muscle samples. Although corresponding muscles on the two sides of the body may agree closely, even adjacent muscles often vary widely in carbohydrate content. Storage in the subcutaneous tissues or in the fluid retained in edema should not be neglected (66). Control experiments demonstrating that this method is capable of accounting for recoveries approaching 100 per cent as in the early work of Folin, Trimble & Newman (66) have not been published. Intermediary steps between glucose in the blood stream and glycogen analyzed in the tissues may play a very important role in obtaining a true balance. The eviscerated preparations usually employed in these studies are very unstable: average blood lactate values of 180 mg.

per cent and rapidly falling glycogen levels illustrate this. With such disintegrative processes the possibility is slight of obtaining a true measure of total carbohydrate present merely by blood lactate and muscle glycogen analyses. That other intermediaries are very likely formed is substantiated by Graeber (74), who found a fall of 300 mg. per cent in lung total carbohydrate, accompanied by an increase of only 75 mg. per cent in lactic acid. In such "balance" experiments, positive conclusions are drawn from negative results—that is, the lack of recovery of any carbohydrate material leads to the conclusion of an increased carbohydrate utilization.

TISSUE OXIDATION

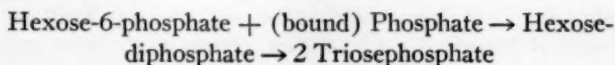
Fermentation scheme.—To facilitate discussion of the work reported on this phase, an outline, admittedly very bare and with many steps omitted, will be used.



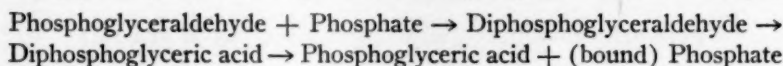
Outstanding has been the work of the Coris, recently summarized for muscle (39, 40), and for liver (41). They have separated the phosphorylase responsible for reaction (a), the phosphoglucomutase responsible for (b), and the phosphatase splitting the hexose-6-phosphate into glucose and phosphate. Reaction (a) has been reversed with liver preparations as well as muscle, but no success has been obtained with the other reactions. Similar results have been obtained as far as concerns the liver by Ostern, Herbert & Holmes (142, 143). They also report the formation of glycogen from the 1-ester (Cori-ester), and have extended the work to the aerobic formation of glycogen from glucose by liver slices. Added phosphate does not seem to be necessary, but calcium must be present. Since the Embden-ester (6-ester) has not been found to yield Cori-ester or glycogen in liver, the authors conclude that their glucose-glycogen synthesis probably passes through the 1-ester, but not the 6-ester. The work from these two laboratories has thrown the emphasis upon phosphorylative rather than upon diastatic breakdown of glycogen in the liver for the maintenance of blood sugar. In muscle, the Embden-ester may be further phosphorylated and enter into a series of reactions finally producing lactic acid.

The formation of glycogen from hexose-1-phosphate was earlier accomplished by Schäffner (150) and by Kiessling (100), using yeast

preparations. The inhibition of phosphorylative glycogenolysis in muscle extracts by the addition of glucose (71, 107) has been extended to liver brei by Soskin *et al.* (161), who suggest that this may be the mechanism which causes the liver to decrease its glucose output in the presence of a high blood sugar. Insulin injected into the animal reinforced the glucose inhibitory effect on brei from excised liver samples (167). Gill & Lehmann have further found that oxidizing agents decrease, and reducing agents increase, the proportion of 6-ester over 1-ester formed from glycogen by muscle extract. Phlorhizin (in high concentrations) increased the carbohydrate content of liver slices, principally by inhibiting its oxidation (4). This may be related to the blocking of glycogen phosphorylation by phlorhizin (41).

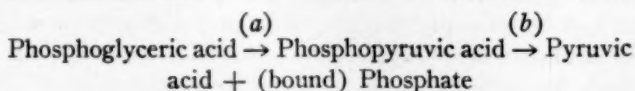


The role of hexosediphosphate in skeletal muscle has been further studied by Deuticke & Hollmann (52). Only by mincing or by chemical poisoning of the muscle can diphosphate be found (cf. 148). Calcium also seems to be concerned (95). Several studies have appeared on the accumulation of hexosediphosphate in glucose fermentation by living yeast (103, 121, 134). Some triosephosphate also was detected.

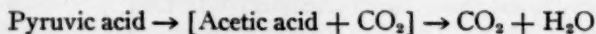


Meyerhof (129) has presented more evidence to substantiate his earlier belief that glyceraldehyde phosphate, rather than dihydroxyacetone phosphate, is the triosephosphate isomer undergoing further degradation. Additional corroboration is furnished from Warburg's laboratory by the elucidation of the intermediate steps in the oxidation of glyceraldehyde phosphate (138, 171). The necessary enzyme components as well as the diphosphoglyceric acid were isolated and purified. Under suitable conditions, the latter compound has been found to transfer one phosphate to adenyldiphosphate.

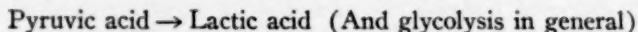
Süllmann believes that the inhibition of lactate formation in muscle by glyceric aldehyde may be due to a condensation between this compound and triosephosphate to form a ketose-1-phosphate (165).



It has been shown (130), by means of radioactive phosphorus, that (b) above is the only irreversible intermediary reaction of fermentation. In oxygen, a reversal can be obtained by the addition of malic or fumaric acid, presumably due to phosphorylation of an oxidation product of the added compound (98).



Lipmann, working with *B. Delbrückii*, has presented the concept that pyruvate is phosphorylated to a labile intermediary (not the usual phosphopyruvate) prior to decarboxylation. The "acetylphosphate" formed then gives up its phosphate to adenylic acid, and the reactive carbonaceous residue may be further oxidized, used for acetylations, etc., (109). However, Ochoa, Peters & Stocken (140) have found that acetylphosphate is not oxidized by brain, and does not phosphorylate adenylic acid in the presence of active muscle extracts. Therefore, this compound may not be related to the labile intermediary noted by Long & Peters (111) in the decarboxylation of all α -ketomonocarboxylic acids by washed brain. In the retinal metabolism of lactate, Grieg, Munro & Elliott (78) observed the intermediate production of an acid (neither pyruvic nor acetic), which was later oxidized. Barron & Lyman (15) contrast the oxidative metabolism of pyruvate with that occurring under anaerobic conditions.



Meyerhof has found that the coupling between the oxidation-reduction of triosephosphate plus pyruvate and the phosphorylation of a suitable acceptor takes place in extracts of brain (128) and embryo (131), as in muscle extract. He has calculated that the speed of the reaction is of the same order as glycolysis in the intact tissues. This fact, plus the necessity of inorganic phosphate, makes it seem probable that the concept of nonphosphorylating brain and embryo glycolysis may have to be modified. In contrast is the conclusion of Geiger & Magnes (68) that brain glycolysis differs in every respect from muscle glycolysis. However, their results strongly suggest some involvement of phosphate. A phosphorylative glycolysis in retina extracts also has been reported (166). Liver brei produces practically no lactate from either glycogen or hexosediphosphate, but the oxidation-reduction reaction between glycerophosphoric acid and pyruvic acid takes place as in muscle brei (79).

Holmes has found that an amount of x-ray irradiation sufficient

to inhibit lactate formation from glucose 50 to 75 per cent has no effect on its formation from hexosediphosphate or hexosemonophosphate (96). Irradiation of mouse kidney with x-ray or radium produced no effects on the oxygen consumption, but the use of unfiltered radon decreased oxygen consumption about 50 per cent, with an even greater fall in anaerobic glycolysis (73). The respiration and anaerobic glycolysis of mouse sarcoma 180 were severely depressed by radon, but the control anaerobic glycolysis of the sarcoma was found to be only of the same order as that of the kidney. In the above reports, interpretation of metabolic changes produced by *in vitro* irradiation of tissues is rendered nearly impossible by the rapidly diminishing activity observed even without special treatment.

Mayer (125) has correlated degree of inhibition of glycolysis of muscle extracts with increasing potentials of several dyes which have no toxic effect (tested in the reduced form). Inexplicable exceptions are also reported.

Relation of oxidative and anaerobic carbohydrate breakdown.—Barker, Shorr & Malam (14) have added to the evidence accumulating that lactic acid formation is not a necessary condition for carbohydrate oxidation. This separation was accomplished in several mammalian tissues by the use of iodoacetic acid, and in brain cortex by prolonged washing in Ringer solution. The separation between oxidative and anoxidative carbohydrate catabolism was placed before the pyruvic (or other acid) stage; Engelhardt & Barchash considered hexosemonophosphate as the last compound common to both paths (58). Ball explained the split purely on the basis of what happens after the formation of reduced diphosphopyridine nucleotide: if aerobic, the hydrogen is oxidized to water through cytochrome-flavo-protein; if anaerobic, the hydrogen is accepted by pyruvic to form lactic acid (6).

Citric acid cycle.—The interest in the Szent-Györgyi theory of hydrogen transport through C_4 acids has been largely diverted to the citric acid cycle theory of Krebs & Johnson (102), which would account for the formation of carbon dioxide as well as water in the oxidation of a three-carbon carbohydrate chain. Although much criticism has been levelled against the latter theory, very few convincing data have been presented on either side. The most important single step, namely, oxalacetic + triose \rightarrow citric acid, has been extensively studied by Breusch (24), who was unable to find citric synthesis by pigeon or mammalian muscle under any circumstances. Kidney was

the only tissue showing a large citrate formation, whereas brain, liver, and lung produced only traces, even in the presence of large amounts of pyruvate and oxalacetate. In contrast, Hallman & Simola have reported a very active synthesis of citrate by minced heart muscle (80).

Thomas (168) has attacked the cycle at several points; however, his strongest evidence, that the keto acid accumulating in the presence of arsenite is pyruvate rather than α -ketoglutaric, has not been substantiated in this laboratory. Mårtensson does not consider that the data on excretion of various intermediaries should be interpreted in terms of a cycle (124). Indeed, Simola's early paper reporting a large increase in urinary α -ketoglutaric and citric acids after pyruvate administration (156), often quoted as substantiating the citrate cycle, must be modified by his later report that sodium bicarbonate, corresponding to the amount of sodium used to neutralize the pyruvic acid, caused fully as large a citrate excretion (157).

The reported deficient succinic oxidase activities in brain (10, 77) and embryo (78), despite their high carbohydrate metabolism, would seem to be evidence against any series of events involving succinate in these tissues. Banga, Ochoa & Peters have indicated that citrate is not involved in brain metabolism (8).

The increased oxygen consumption of pigeon muscle brei caused by insulin *in vitro* (101) has been confirmed (155, 162), as well as extended to pigeon brain (9), but has not been found in mammalian cardiac or skeletal muscle (155). Furthermore, the insulin effect in minced pigeon muscle has even been found in the absence of added citrate (162).

REVIEWS

At the end of the bibliography are included several references to review articles dealing with various aspects of carbohydrate metabolism. Since limitations of space do not permit a discussion of the rapidly growing study of the metabolism of the developing embryo, a series of papers appearing from Needham's laboratory have also been included to indicate the type of recent advances in this field.

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THE METABOLISM OF PROTEINS AND AMINO ACIDS

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In the preparation of this review, in which, of necessity, selected topics in the field of the metabolism of the proteins and amino acids are presented, the authors have been handicapped somewhat by the inability to obtain recent European journals. Only those papers which were available and which could be read in full in their original form have been discussed; for this reason, many contributions of undoubted importance which were obtainable only in abstract have been omitted. It is regretted that this has not permitted us to give full recognition to research published in Russia and Japan, as well as in some of the Balkan countries. Within the limitations of the space available, it has not been possible to present as full bibliographic citations as desired nor to discuss many topics of interest. An attempt has been made to include under each of the more important subdivisions, one or more papers which afford extensive general discussions and detailed bibliographic references. Finally it should be noted that throughout this review, we have employed the Fischer-Wohl-Freudenberg nomenclature for amino acids in which the prefixes, *d* and *l*, are used to denote chemical configuration and not the direction of optical rotation.

GENERAL ASPECTS OF AMINO ACID METABOLISM

Investigations using isotopes.—An outstanding contribution is the extension of the application of the heavy isotope of nitrogen to metabolism studies of amino acids. Schoenheimer and his collaborators have described in detail their methods of analysis and the application of the mass spectrometer to the determination of the relative proportions of the isotopes N^{14} and N^{15} in biological materials (1, 2). By the use of slightly modified, standard methods of synthesis, the heavy isotope of nitrogen has been introduced into the α -position of a number of amino acids (3). These preparations contained an average of 1.9 atom per cent excess of N^{15} , and, in an important experiment preliminary to the metabolism studies, it was demonstrated that

there is no *in vitro* exchange of the N^{15} atom of the amino acids with the N^{14} of other nitrogenous compounds when they are heated together in aqueous solution at 100° (4). The suitability of these amino acids for investigations of protein metabolism was thus established.

In a limited number of experiments with rats the utilization of dietary ammonia has been demonstrated (5). The heavy isotope of nitrogen was fed in the form of ammonium citrate. The glycine, proline, histidine, arginine, aspartic acid, and glutamic acid, isolated from the tissues of these animals, contained small but definite excesses of the marked nitrogen. Creatine likewise was shown to have exchanged a small amount of ordinary nitrogen for the heavy isotope. The incorporation of sodium benzoate into the diet demonstrated that at least a small portion of the dietary ammonia could be used for the synthesis of glycine, since the excreted hippuric acid contained a definite excess of the N^{15} . These experiments with ammonia containing N^{15} were later repeated under more nearly physiological conditions and essentially the same results were obtained (6). It is particularly significant that heavy nitrogen, fed in the form of an ammonium salt, was not only introduced into the amino acids, but that the latter compounds were rapidly built into the tissue proteins. These facts indicate a lability of the protein structures of the body that was not to be anticipated from previous investigations.

Rittenberg & Schoenheimer (7) have studied the detoxication of benzoic acid with N^{15} glycine. It is well known that the administration of this amino acid will increase the tolerance for benzoic acid (8) but it is now established that only a small fraction of the dietary glycine is used directly for hippuric acid synthesis. The excreted hippuric acid contained a relatively small portion of the labeled nitrogen and the bulk of the glycine was furnished by the tissues.

The role of tyrosine in metabolism was investigated by the use of the N^{15} *dl*-form of this amino acid (9). Approximately 50 per cent of the administered N^{15} was recovered in the urine of the animals and the remainder was found in the tissues. The heavy isotope could be located in at least four sites: (a) in the α -amino position of the monoamino acids; (b) in the histidine from muscle and liver protein; (c) in the arginine isolated from the liver and carcass of the animals; and (d) in the "amide nitrogen" fraction of the carcass proteins. The isolated histidine and arginine were further investigated with respect to the location of the N^{15} atom in the molecule. It was demonstrated

that the isotope had entered the guanido group of the arginine, a group which represents potential urea (10). The residual ornithine contained no excess of the labeled nitrogen. On the other hand, the marked nitrogen which had entered the histidine molecule was entirely in the α -amino position (11).

In a relatively recent report, Schoenheimer, Ratner & Rittenberg (12) have confirmed and extended their findings by the use of a preparation of *L*-leucine in which was incorporated not only the marked nitrogen but in which the carbon chain was labeled with deuterium. The results of the metabolic studies were, in general, qualitatively the same as were found in the experiments with N^{15} -tyrosine. The use of the deuterium in addition to the marked nitrogen, however, permitted a somewhat more quantitative assay of the fate of the amino acid. The experimental periods were of but three days' duration; nevertheless, heavy nitrogen was found to be widely distributed throughout the body in the amino acids of the tissue proteins. As was to be expected, the N^{15} content of the leucine of the tissue proteins was considerably greater than that of the other amino acids. That a certain portion of the dietary leucine had been introduced directly into the body proteins was proved by the isolation, from these sources, of *L*-leucine which contained both the labeled nitrogen and the deuterium. However, examination of the ratios of D to N^{15} in the tissue leucine fractions indicated that over one-third of the marked nitrogen in the dietary leucine had been replaced with ordinary nitrogen.

It is probably significant that in all of the experiments reported by Schoenheimer and his co-workers, relatively large amounts of N^{15} were found in the glutamic and aspartic acid fractions of the amino acids. This finding is interpreted in support of the present concepts of the importance of the dicarboxylic amino acids in protein metabolism as evidenced particularly by the *in vitro* experiments of Braunstein & Kritzmann (13, 14). It is also interesting that in none of the experiments reported was lysine found to have participated in the exchange of the amino groups, a finding quite in agreement with the earlier work with deuterium (15). It therefore seems that lysine is unique among the amino acids studied in that its deamination product can not undergo reamination *in vivo*.

Bloch & Schoenheimer (16) have reinvestigated the problem of the origin of creatinine. When creatine, prepared from cyanamide and N^{15} -sarcosine, was fed to rats, the heavy isotope of nitrogen ap-

peared very soon in the creatinine of the urine. The amount of the labeled nitrogen excreted at once, however, was small in comparison to the amount in the diet and examination of the tissues revealed an almost uniform distribution of the marked nitrogen throughout the creatine of the entire animal body as well as in the urinary creatinine. The feeding of creatine for a single day, followed by a period in which no creatine was given, led to excretions of creatinine having a content of N^{15} exactly comparable to that of the tissue creatine. The results are believed to have demonstrated that the urinary creatinine is derived solely from the creatine of the body and, since the N^{15} creatinine was rapidly excreted, that the reaction, $\text{creatine} \rightleftharpoons \text{creatinine}$, is probably not reversible in the animal organism. In contrast to the experiments with the N^{15} ammonia and with the N^{15} amino acids, none of the N^{15} of the dietary creatine could be detected in the amino acids of the tissue proteins.

Preliminary data upon the role of glutathione in animal metabolism have been obtained by Waelsch & Rittenberg (17). Within two hours after the administration of N^{15} glycine, an appreciable portion of this amino acid had entered into glutathione combination. This finding indicates a very rapid synthesis and perhaps a rapid breakdown of the tripeptide; the physiological significance of this rapid turnover must await further investigation.

In a summary of the work of his group, Schoenheimer (12) emphasizes the apparent lability of the tissue proteins. Since dietary nitrogen is rapidly incorporated in the protein molecules of the body—a process which demands the opening and closing of peptide bonds—it appears that the nitrogenous groupings of these proteins are constantly involved in a variety of chemical reactions and that the concepts of endogenous and exogenous metabolism are no longer to be considered accurate.

The excreted nitrogen may be considered as a part of the metabolic pool originating from interaction of dietary nitrogen with the relatively large quantities of reactive tissue nitrogen.

The optical inversion of stereoisomerides.—Du Vigneaud & Irish (18) demonstrated that the administration of *d*-phenylaminobutyric acid was followed by the excretion of acetyl-*l*-phenylaminobutyric acid in the urine. In a similar series of experiments, an inversion, *in vivo*, of *S*-benzyl-*d*-cysteine and *S*-benzyl-*d*-homocysteine was shown to take place, these compounds being excreted as the acetyl

derivatives of S-benzyl-*l*-cysteine and S-benzyl-*l*-homocysteine respectively (19). The phenomenon of inversion of an amino acid in the animal body has now been investigated by use of the heavy isotopes of hydrogen and nitrogen (20). *d*- and *l*-Phenylaminobutyric acids were synthesized in which the α -amino group was marked with N¹⁵. The compounds were fed to rats in a fluid diet which was adjusted to a concentration of 4 per cent of deuterium oxide. The administration of the *d*-phenylaminobutyric acid resulted in the excretion of acetyl-*l*-phenylaminobutyric acid; this derivative, however, contained only 1 to 2 per cent of the dietary, marked nitrogen. A considerable quantity of deuterium had been substituted in the α -position. These transformations may be explained by the supposition that the substance fed had undergone deamination, with loss of the heavy isotope of nitrogen, and asymmetric resynthesis by which ordinary nitrogen was introduced into the molecule. Assuming that the first step in the latter process is the amination of the α -keto acid, the presence of the deuterium in the molecule must be accounted for by either a reduction of the imino acid or by a hydration (compare the subsequent discussion of α -ketoglutaric acid). The *l*-phenylaminobutyric acid was excreted as the acetyl derivative in which a considerable portion of the labeled nitrogen had remained attached to the original molecule. In this case, as before, deuterium was found to have been introduced into the compound at the α -position. When acetyl-*d*-phenylaminobutyric acid was fed it was excreted unchanged and contained no deuterium atoms on the carbon chain. It is believed that these interesting results permit the conclusion that the *d*-amino acid is more readily deaminated in the animal body than is the *l*-form; since the α -keto acid is believed to be in equilibrium with the α -imino acid (21), the transformations described are in good accord with the results of recent enzyme investigations concerned with the metabolism of amino acids (13, 21). The acetylation probably resulted from condensation of the intermediate imino acid with pyruvic acid, a transformation in harmony with the results of the reactions of transamination which are discussed in a later paragraph.

In view of certain implied differences in the utilization of tryptophane by the mouse and the white rat (22), Totter & Berg (23) have investigated the growth-promoting properties of the *d*-forms of tryptophane, histidine, and lysine in the mouse. The unnatural forms of tryptophane and histidine are capable of supporting growth when added to diets deficient only in these two amino acids; they are, how-

ever, less efficient in this respect than are the corresponding naturally occurring forms. Unnatural lysine in the mouse, as in the rat, fails to promote growth when added to diets deficient in the natural form of this amino acid.

The occurrence of d-glutamic acid.—The occurrence of *d*-glutamic acid in the culture media of certain bacteria was reported several years ago (24, 25). Recently Kögl & Erxleben (26) have reported the isolation of the *dl*-form of this amino acid from tumor tissue. Since the methods used precluded racemization during the isolation, the authors believed that the presence of *dl*-glutamic acid was a chemical characteristic of malignant tissue. These results were confirmed by Arnow & Opsahl (27) and by White & White (28). On the other hand, Graff (29) and Chibnall and co-workers (30) reported that they were able to obtain only the *l*-form of the amino acid. Kögl & Erxleben (31) pointed out that the methods used by Chibnall were not suited to the isolation of the *d*-form and re-emphasized their original contentions with respect to the racemic nature of the glutamic acid in tumor tissue. Recently Johnson¹ has obtained the *d*-glutamic acid from normal rat liver and this has been confirmed in the same laboratory by White who has worked with human liver. Chibnall has likewise found that the *d*-glutamic acid is not unique as a constituent of tumor tissue, but has obtained the compound from a number of plant proteins as well as from normal animal tissues. This form of the amino acid is, therefore, probably much more widely distributed in nature than had been supposed.²

Transamination.—The reviews of the past two years³ have mentioned briefly the results of the investigations of Braunstein & Kritzmann (13, 14) upon the intermolecular transfer of amino groups between α -amino acids and α -keto acids. As most of the recent work

¹ The reviewers are indebted to Dr. J. White for the information concerning the unpublished findings of Johnson, White, and Chibnall.

² A recent, preliminary report (32) upon the application of the *d*-amino acid oxidase system to the determination of the *d*-amino acid content of proteins has indicated that from 0.6 to 3.7 per cent of the total nitrogen is present in the form of the *d*-amino acids. The proteins studied were prepared from normal and tumor tissues and included also insulin and Bence-Jones protein. The results confirm the view expressed above that, regardless of the general biochemical interest in the occurrence of the *d*-amino acids as constituents of protein, the contention of specificity in malignancy is not valid.

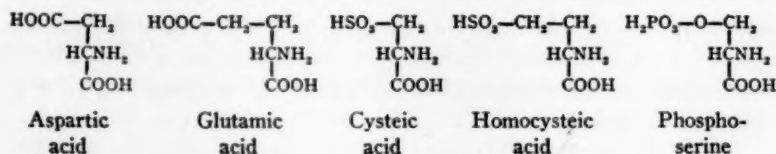
³ *Ann. Rev. Biochem.*, **7**, 189 (1938); **8**, 249 (1939).

has been available only in the form of short notes (33, 34, 35), the more detailed summary of Braunstein in German (36) is a welcome contribution.

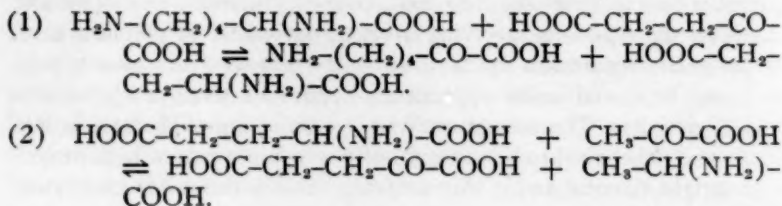
Fundamentally, the reaction of transamination involves the transfer of an amino group from an α -amino acid to an α -keto group of a dicarboxylic acid (usually either α -ketoglutaric acid or oxalacetic acid). The reaction is reversible and reaches an equilibrium at a very rapid rate. The enzymes responsible for the transamination are apparently two in number. The first, termed glutamico-aminopherase, catalyzes the reactions involving α -ketoglutaric acid or glutamic acid. It has been prepared in the form of a dry powder from muscle pulp and may be stored under appropriate conditions without appreciable loss of activity. The second enzyme, aspartico-aminopherase, is less stable and is believed to be made up of two components, a high molecular weight enzyme and a thermostable, readily diffusible coenzyme. This system is responsible for the transaminations which involve aspartic acid or oxalacetic acid.

The enzymes are apparently widely distributed. The first observations were carried out with muscle tissues, but the processes of transamination were detected in brain, kidney, liver and heart tissues. It is now proved that intestinal mucosa contains the enzymes but that they are not present in glandular tissues, stomach, or lung. The reactions of transamination have been demonstrated in tumors (35, 37), in microorganisms (38, 39), and in plant tissue (40).

Although it was originally believed that the transfer of an amino group required the participation of either an amino- or a keto-dicarboxylic acid, it has now been shown that the specificity of the reaction is governed by the substituent groups of the molecule (36). Thus, in the presence of pyruvic acid and macerated muscle tissue, not only aspartic acid and glutamic acid are able to transfer their amino groups, but cysteic acid, homocysteic acid, and phosphoserine likewise are able to convert the pyruvic acid into alanine. The chemical relationship between these compounds is evident from their formulae:



The direct transfer of the amino group from a monoamino-monocarboxylic acid to a keto-monocarboxylic acid has not been observed (36). This type of reaction, however, has been brought about by the use of a dicarboxylic acid derivative as an intermediate carrier. Thus, under the influence of the glutamico-aminopherase and in the presence of glutamic acid or α -keto-glutaric acid, lysine will transfer its amino group to pyruvic acid. The suggested role of the dicarboxylic acid is as follows:



The reactions of transamination are probably specific for the *l*-forms of the amino acids. The alanine formed by the amination of pyruvic acid in the presence of the donor of an amino group has been proved to be the *l*- or natural form. In the presence of broken cell suspensions, the *d*-forms of certain amino acids act as donors of amino groups to a very slight extent, but in the presence of the isolated enzyme systems they are without this action.

Although the experimental obstacles associated with the work are difficult to surmount, evidence of a transamination, *in vivo*, has been presented (36). Injection of glutamic acid into rabbits was accompanied by a marked and rapid increase in the concentration of free alanine in the muscle tissue and the injection of α -ketoglutaric acid into mice brought about a two- to three-fold increase in the free dicarboxylic-amino-nitrogen of the tissues. The experiments, admittedly imperfect, disclose the possible operation of the transaminating enzymes within the animal body, a possibility, in part substantiated by the work of Schoenheimer and his associates (9, 12).

Cohen (41) has applied his new method for the micro-determination of glutamic acid to a study of the transaminating system of pigeon muscle and although he was able to confirm the findings of Braunstein & Kritzmman with respect to alanine he was unable to demonstrate an appreciable rate of transfer of amino groups from other amino acids.

Knoop & Martius (42) have studied the synthesis of octopine,

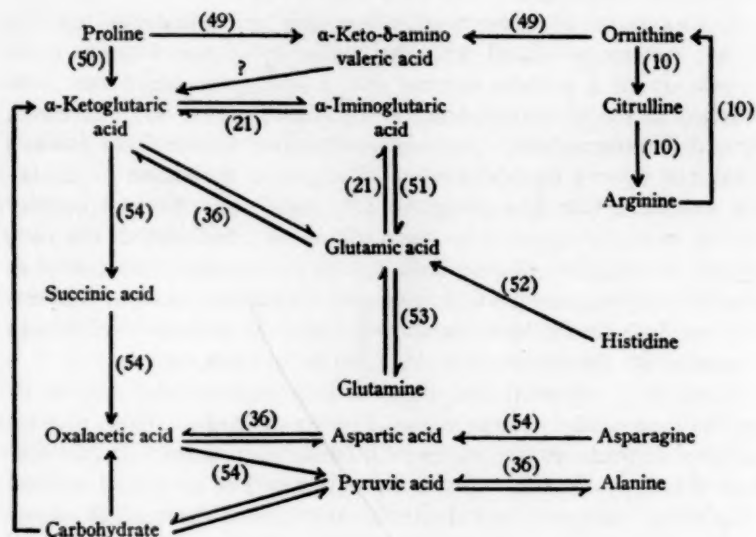
N^ε-(1-carboxyethyl) arginine, by the catalytic reduction of the Schiff's base formed from pyruvic acid and arginine. The authors believe that the biological function of octopine may be that of an intermediate compound, important in amino acid synthesis. It is further suggested that arginine may function *in vivo* as a transaminating agent, similar in behavior to glutamic and aspartic acid. No biological studies have been reported in support of this view. However, in this connection it is of interest to point out that Irvin & Wilson (43) have shown that during autolysis of the adductor muscle of the scallop (*Pecten magellanicus*), arginine disappeared and evidence for the formation of octopine was obtained. The rate of formation of octopine was more rapid in sliced than in hashed muscle.

The oxidation of amino acids.—It is now well established that the enzyme system associated with the oxidation of the *d*-amino acids is made up of a protein enzyme and a coenzyme which has been identified as the alloxazine-adenine dinucleotide (44, 45). Axelrod, Sober & Elvehjem (46) have shown that the tissues from animals maintained upon a diet deficient in riboflavin, a derivative of alloxazine, exhibit a low rate of amino acid oxidation. Normal activity was but partially restored by the addition of riboflavin to the diet, whereas the addition of both riboflavin and meat extract (designated as factor W) regenerated the full activity of the oxidase system. The authors conclude that at least one dietary factor in addition to riboflavin is essential for the synthesis, *in vivo*, of the enzyme system.

Klein (47) reported that there is a decrease in the content of *d*-amino acid oxidase in the tissues of thyroidectomized rats and an increase in content in the tissues of animals maintained upon an adequate diet supplemented with thyroid tissue. The increased activity in the latter instance is not due to an increase in the content of the coenzyme in the tissues, but is probably associated with an increased concentration of the specific protein portion of the enzyme system (48).

Krebs (49) has shown that the *d*-amino acid oxidase system acts upon *d*-proline with the formation of α -keto- δ -aminovaleric acid. These results suggested that *d*-ornithine should yield the same derivative of valeric acid, and this was experimentally proved. These findings are of interest in that they further extend the interrelations between the amino acids which have now been established by the work with tissues and tissue extracts. These are graphically illustrated in the chart adapted from Krebs' report (p. 286).

The metabolism of α -ketoglutaric acid.—It has been known for some time (55) that the oxidation of glutamic acid in muscle yields succinic acid, probably by way of the intermediates, α -iminoglutaric acid and α -ketoglutaric acid. Krebs & Cohen (51) have now shown that the oxidation of this keto acid by heart and kidney tissue is probably a dismutation in which a molecule of the keto acid reacts with ammonia to form α -iminoglutaric acid. This derivative undergoes an intermolecular dismutation with a second molecule of α -ketoglutaric acid yielding succinic acid and glutamic acid. The α -iminoglutaric acid thus acts as a hydrogen carrier for the oxidation of the α -keto acid.



N-Alkyl derivatives of amino acids.—N-Monomethyl derivatives of certain naturally occurring amino acids have been observed to be readily available for the promotion of growth of young white rats maintained on inadequate diets, the limiting factor of which was the shortage of the specific essential amino acid whose N-methyl derivative was under study. To those previously listed⁴ may now be added the α -N-monomethyl derivatives of *L*-cystine (N, N'-dimethylcystine)

⁴ *Ann. Rev. Biochem.*, **8**, 260-1 (1939).

(56), *l*-phenylalanine (57) and *l*-tryptophane [1(+)-abrine] (58). On the other hand, the corresponding derivatives of *dl*-lysine (59), *dl*-valine (57), *dl*-leucine (57), *dl*-isoleucine (57), *d*-cystine (56), and *d*-phenylalanine (57) were not utilized for growth. It is notable that with the exception of the derivative of *l*-cystine, the N-monomethylamino acids which can replace the corresponding amino acids are those whose unnatural enantiomorphs are also utilized. On the other hand, *d*-phenylalanine and *d*-tryptophane promote growth while the N-monomethyl derivatives of the *d*-amino acids are not effective (57, 58) in this respect. It is suggested that the monomethyl compounds undergo oxidative deamination and that the α -keto acid is available for amination. Methylation of the amino acid of the unnatural series appears to prevent this oxidative deamination. The α -keto acid (*p*-methoxyphenylpyruvic acid) has been isolated from the urine of rabbits fed O, N-dimethyltyrosine (60).

Both sarcosine (N-methylglycine) and glycine increased the rate of excretion of hippuric acid by the rabbit after the administration of benzoate, although the effect of sarcosine, while marked, was not as great as that of glycine (61). This was interpreted as evidence of the conversion of sarcosine to glycine. Neither N, N-dimethylglycine nor the completely methylated glycine derivative, betaine, increased the rate of excretion of hippuric acid under these conditions. This evidence of the relative stability of the dimethyl and more highly methylated derivatives is in agreement with the suggestion that for oxidative deamination of N-substituted amino acids to occur, at least one hydrogen must be attached to the nitrogen of the amino group.⁵

Further support of this theory is to be found in the failure of α -N, α -N-dimethyl-*dl*-lysine (59) and N, N-dimethyl-*dl*-phenylalanine (57) to promote growth in lieu of the unsubstituted amino acids. The failure of acetyl-*l*-N-methyltryptophane to be utilized for growth is in sharp contrast to the positive results obtained with both acetyltryptophane and N-methyltryptophane (62) and is further evidence of the necessity of the presence of at least one unsubstituted hydrogen in the α -amino group for the occurrence of oxidative deamination of the N-substituted amino acids. N-Ethyl-*dl*-phenylalanine is not utilized for growth of the white rat (57) suggesting that N-alkyl groups other than methyl are not readily cleaved from the amino acids. α -N-Monomethyltryptophane (abrine) is demethylated and metabo-

⁵ *Ann. Rev. Biochem.*, **5**, 250 (1936).

lized and appears in the urine as kynurenic acid and kynurenin after administration to rabbits (63). It is also effective in promoting recovery from phenylhydrazine anemia in rabbits (64).

THE ESSENTIAL AMINO ACIDS

The study of the role of the individual amino acids and their simple derivatives in the promotion of growth of the young white rat has continued in the laboratory of Rose, who has presented excellent reviews of the general problem (65, 66). Valine, an amino acid relatively simple in its structure, must be present in adequate amounts in the diet of the young white rat (67). Animals which were fed diets whose nitrogen was derived from a mixture of highly purified amino acids but which contained no valine, experienced profound nutritive failure, manifested by decreased food consumption, rapid loss in weight, and eventually death. Characteristic symptoms of the valine deficiency included extreme sensitivity to touch and a severe lack of coordination in movement. It is noteworthy that all of the forked chain aliphatic amino acids known to occur in the protein molecule have now been demonstrated to be essential for the growth of the young white rat.

Adult female white rats were fed a diet low in its content of nitrogen. The diet was inadequate for the maintenance of nitrogen equilibrium as evidenced by marked negative nitrogen balances. When a mixture of the nine amino acids commonly listed as essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine) was added to the basal diet, loss of body nitrogen was prevented and positive nitrogen balances were observed (68, 69). Omission of any one of these amino acids from the supplementary amino acid mixture resulted in a negative balance of nitrogen, while subsequent replacement was followed by a restoration of nitrogen equilibrium.

Previous studies of the essential nature of the amino acids in which mixtures of highly purified amino acids have served as the sole source of nitrogen have been limited to growth experiments with young white rats. Similar experiments with dogs, in which nitrogen balances have been determined (70), have shown that the amino acids which are dispensable for the growing rat are also dispensable for the adult dog, but that for the adult dog, arginine is not a necessary dietary component. The significance of these findings lies in

The fact that two widely different species require for their well-being the same components of the protein molecule, increases the probability that other mammals, including man, may manifest like responses.

THE INTERMEDIARY METABOLISM OF THE INDIVIDUAL AMINO ACIDS

Glycine and alanine.—The intermediary metabolism of glycine, the simplest of the amino acids of the protein molecule, is still obscure. When glycine was administered orally (71, 72) or intraperitoneally (71) to fasted white rats, the content of liver glycogen was not increased significantly.* These results were in sharp contrast to the marked increases of liver glycogen noted after the similar administration of alanine. The failure of glycine to spare protein when fed to fasting dogs and the protein-sparing action of alanine (71) suggest that alanine, but not glycine, may form sugar in the living animal under conditions of carbohydrate deprivation and that the metabolic pathways of the non-nitrogenous residues of alanine and glycine are different. Although formation of carbohydrate from non-carbohydrate precursors was observed when liver slices of fasted rats were shaken *in vitro* with a number of amino acids (including alanine), the presence of glycine resulted in no increased carbohydrate formation and, in some experiments, actually led to a diminished glycogenesis (74). These results are not in accord with the generally accepted belief that glycine is a glucose former as evidenced by the excretion of extra glucose in the phlorhizinized dog after the administration of the amino acid.

When glycine is administered to the intact living animal, deamination with the subsequent increased production of urea occurs readily. On the other hand, in experiments *in vitro* it is difficult to demonstrate deamination of glycine. The contradictory nature of the evidence is well summarized by Bach (75). Glycine, unlike alanine and many other amino acids, is deaminated little, if at all, when incubated with tissue slices of the organs usually studied in experiments of this sort. Deamination was not increased when ornithine was added as a "fixative" for any ammonia formed, nor was the formation of either ammonia or urea observed when cat liver or kidney was perfused with glycine (75). Bach suggests that condensation with biologically important groups (e.g., α -keto acids) is more significant in the inter-

* Evidence of the formation of glycogen from glycine is afforded by the work of Butts and co-workers (73).

mediary metabolism of glycine than is simple deamination. Such condensation may permit glycine to

act as fixative for keto compounds producing an equilibrium effect in systems responsible for the formation of such compounds. The latter phenomenon may lead to a basis of the well-known "specific dynamic effect" of glycine by picturing its rôle as an acceptor of aldehydes and keto products thus catalysing the reaction within the enzymic systems concerned. By its condensing capacity it may act as a general reagent for the removal of compounds the metabolism of which would otherwise come to a standstill.

Glycine is not essential for growth in the young white rat (65). Glycine in large amounts (4 gm. daily) and alanine are reported to be toxic to hens (76) which are not able to use glycine for the detoxication of sodium benzoate. In rabbits the limiting factor in the detoxication of benzoic acid and in the excretion of the conjugated product, benzoylglycine (hippuric acid), appears to be the availability of glycine. While glycine may be synthesized in response to its need in the detoxication reaction, an increased rate of synthesis and excretion of hippuric acid is observed when an abundant supply of exogenous glycine is furnished. Sarcosine (N-methylglycine) may give rise to glycine for hippuric acid synthesis (61). It seems improbable that this is an important source of glycine in the body normally, although muscle creatine is a potential precursor of sarcosine. The determination of the creatine content of muscle tissue by the newer and more highly specific methods, after repeated administration of benzoic acid, should prove of interest in this connection. Much of the available evidence suggests that glycine may be a factor in the synthesis of creatine and creatinine (77, 78), but the reverse reaction by which these compounds would yield glycine biologically remains to be demonstrated.

The synthesis of hippuric acid by the liver and kidney of the more common laboratory animals (in the dog, kidney only) has been effected from benzoic acid and glycine in tissue slice experiments (79). A substance which behaved like hippuric acid in the usual analytical procedures for hippuric acid was formed when serine or hydroxyproline was used, although synthesis proceeded more rapidly with glycine.⁷ All other amino acids, including threonine, gave negative results. The

⁷ Since this review was submitted for publication, details of these studies have become available [*J. Biol. Chem.*, **132**, 307 (1940)]. The conclusion is reached that only glycine led to the formation of hippuric acid by rat liver slices.

behavior of threonine and of serine is of especial interest in view of the suggestion that the hydroxyamino acids are potential precursors of glycine in the biological synthesis of this amino acid.* Full details of these experiments should prove of unusual interest.

The importance of an adequate supply of dietary carbohydrate in the promotion of hippuric acid synthesis, either by facilitating the synthesis of glycine or its conjugation with benzoic acid, has again been emphasized (80).

In confirmation of the work of Corley and others,⁹ Polonovski & Boulanger (81) have shown that if the hydrogen attached to the α -carbon of an α -amino acid is substituted by a methyl group, as in α -methylalanine, deamination does not take place in the normal manner.

The aromatic amino acids and their derivatives.—The production of experimental alcaptonuria in white rats by feeding large amounts of phenylalanine has been demonstrated (82, 83, 84). These findings have now been extended to two other species, the guinea pig and man (85). When guinea pigs on diets deficient in ascorbic acid received orally 0.5 gm. of *l*-tyrosine daily, 20 to 50 per cent of the theoretical amount of homogentisic acid (determined colorimetrically by the Briggs method and identified by the isolation of the dibenzoylhomogentisamide) was excreted in the urine (85). Administration of 5 mg. of ascorbic acid resulted in a prompt disappearance of the alcaptonuria. Similar observations were made with two normal human subjects. These data are of interest not only because of the suggested relation of ascorbic acid to the oxidation of the phenyl nucleus of the naturally occurring amino acids but also because alcaptonuria has not been produced in rats by the administration of tyrosine (82, 83). The failure to obtain excretion of homogentisic acid in rats when the phenylalanine was fed as the hydrochloride, or in the presence of sodium carbonate at a pH of 8, is difficult of explanation (84) as are also the variable excretions of phenylpyruvic acid and phenylalanine under similar experimental conditions (86). The production of phenylpyruvic acid from *l*-phenylalanine has been shown to be comparatively common in bacterial cultures (87) notably in those of the various types of *Bacillus proteus* and of Morgan's bacillus. Whether this activity of microorganisms is related to congenital phenylpyruvic oligophrenia (Fölling's disease) is not known.

* *Ann. Rev. Biochem.*, **6**, 270 (1937).

⁹ *Ibid.*, **7**, 191 (1938).

Further evidence of the relation of ascorbic acid to the metabolism of the aromatic acids has been obtained in a preliminary study of the urine of premature infants (88) which received cow's milk. These urines gave a strong Millon's reaction which was not due to the presence of tyrosine, dihydroxyphenylalanine, homogentisic acid, or melanin. *l-p*-Hydroxyphenyllactic acid was isolated and identified. The excretion of total hydroxyphenyl compounds (calculated as tyrosine) was approximately 1.0 gm. in two of the cases studied. Administration of 50 to 200 mg. of ascorbic acid resulted in a prompt diminution in the amount of aromatic acid in the urine.

The isolation of thyroxine from iodinated casein by Ludwig & Mutzenbecher (89), which has been confirmed by Harington & Rivers (90), raises anew the problem of the biological synthesis of this iodinated derivative of the naturally occurring aromatic amino acid. Two moles of diiodotyrosine may couple oxidatively with the elimination of one side chain. In the artificial synthesis of thyroxine from casein by the action of iodine, it has been suggested that under the experimental conditions (37°, pH 8.5, four-hour period) the iodine may be able to effect this oxidative coupling (89). Harington & Rivers (90), however, consider it improbable that iodine could bring about so drastic an oxidation or that tyrosine in combination, as in the protein molecule, could react thus. The hypothesis is advanced that casein (and if this is a mechanism of biological synthesis, probably other proteins) may contain preformed thyronine which is iodinated to yield thyroxine, although it is admitted that thyronine has hitherto escaped detection in proteins; also the conversion of thyronine to thyroxine has not yet been accomplished.

The 3,5-diiodo-4-hydroxyphenyl ether of thyroxine in which three diiodohydroxyphenyl groups are linked in a chain to α -amino-propionic acid has been synthesized and demonstrated to be without significant physiological activity (91).

Tryptophane.—The study of the excretion of kynurenic acid as an end product of tryptophane metabolism by various species of carnivora has been undertaken to secure further data as to possible correlation between the excretion of this acid and zoological classification. After administration of tryptophane, kynurenic acid was present in the urine of the hyena, coyote, wolf, fox, and badger and was absent from the urine of the serval, cheetah, civet, genet, bear, racoon, and sea lion (92). The data are compatible with the hypothesis that the excretion of kynurenic acid, or its failure to be excreted, may

be characteristic of the species which comprise each family of the carnivora.

*The sulfur-containing amino acids.*¹⁰—The indispensability of methionine and the synthesis of cystine by the living organism (65, 66) have received further confirmation (93). Young white rats were fed diets in which a casein hydrolysate from which cystine had been precipitated served as a source of nitrogen. At the end of six weeks, during which fair growth was observed, the total cystine of the carcasses was markedly in excess of that present in control rats killed at the beginning of the experimental period. Since the diet was essentially cystine-free, the cystine used for the building up of new tissue may be assumed to have been synthesized from methionine which is present in casein in considerable amounts (3.2 per cent). In other experiments in which young white rats were fed labeled methionine containing radioactive sulfur (S^{35}), the presence of the radioactive isotope could be demonstrated in the cystine isolated from the hair and skin of the animals (94). Since the sulfur of cysteine has been shown to be stable and not to exchange with S^{35} present in hydrogen sulfide (95), these studies are believed to support the theory of a direct conversion of methionine to cystine without cleavage of a sulfur-containing nucleus.

The ability of the young white rat to utilize dietary homocystine (or homocysteine) in lieu of the essential methionine appears to be associated with secondary dietary factors other than the amino acid (96, 97). When the vitamin-B supplement was supplied by the various components which are available in pure form, utilization of homocystine did not occur, while when the B complex was furnished by milk concentrate and tikitiki (rice-bran concentrate), positive results were obtained. It now appears that the unknown factor is choline, which has been isolated from these vitamin concentrates in significant amounts (98). When choline was fed with homocystine, the rat was able to utilize homocystine for purposes of growth in lieu of methionine. The observation that betaine may also serve as a source of methyl groups for the synthesis of methionine when fed with homocystine (98) is of particular interest in view of the commonly accepted belief (based on inadequate experimental data!) in the biological inertness of this completely methylated glycine derivative. These observations serve to emphasize anew the fact that

¹⁰ See also pp. 199-230.

until all of the "accessory substances" are available in crystalline form, one should not overlook the possibility of the presence in the food of contaminants which may be instrumental in the synthesis by the organism of essential dietary components (96).

When diets low in their content of choline were fed to young white rats, hemorrhagic lesions of the kidney developed in about ten days (99). The presence of cystine in the diet increased this effect while methionine delayed its onset or prevented it entirely. These observations and those which concern the relation of choline, cystine, and methionine to the dietary production of fatty livers,¹¹ suggests a relationship, as yet not clearly defined or explained, between the metabolism of choline and that of the sulfur-containing amino acids.

The favorable effect of methionine as a supplement to peanut-meal protein in promoting lactation in rats and the absence of such an effect with supplementary cystine (100) are further evidence of the difference in the biological roles of the sulfur-containing amino acids.

Cystine is important in serum protein regeneration in dogs, in which hypoproteinemia has been produced and maintained by the combination of plasmapheresis and a low protein diet (101). When gelatin is supplemented by cystine and either tryptophane or tyrosine, 25 to 40 per cent of the protein content of the combination is converted to plasma protein, an efficiency equal to that of any protein hitherto tested. Methionine can not substitute for cystine nor phenylalanine for tyrosine in the combination gelatin-cystine-tyrosine. The cystine content of serum proteins which is greater than that of the majority of the proteins except the keratins should be recalled in the consideration of these experiments.

More recent studies have confirmed (102) the earlier observations that cystine (103) and methionine (104) do not give rise to glycogen in the liver. Stöhr (72) had previously reported that cystine was a glycogen former. The failure to observe glyconeogenesis by this method is not in accord with the observed excretion of "extra" glucose after cystine or methionine feeding in phlorhizin glycosuria. A repetition of the phlorhizin experiments should be of interest.

The basic amino acids and related compounds.—The specificity of the occurrence of histidinuria in the diagnosis of pregnancy has not been confirmed. In a study of 300 patients in which 669 specimens of urine were examined (105), histidinuria was found in both males

¹¹ *Ann. Rev. Biochem.*, **8**, 351 (1939).

and females, in health and in disease. While histidine was usually present in the urine of pregnancy, this finding can not be relied upon as diagnostic of this condition. An excretion of 100 to 200^{mg.} of histidine per day has been observed in the urine of normal adults (106). Since the method used involved losses, it is estimated that the daily excretion is, at least, 300 mg.

Methods for the quantitative determination of carnosine and anserine have made possible the study of the distribution in muscle of these interesting dipeptides which contain histidine or methylhistidine (107). Anserine was present in greater concentrations than carnosine in all mammalian muscles studied except those of the horse and ox. An excellent review of the chemistry and physiology of carnosine and anserine is available (108).

After oral or intravenous administration to man and the rabbit, *l*(+)-arginine disappeared rapidly from the serum (109) and the presence in the serum of a protein abnormally rich in arginine could be demonstrated within one to two hours (110). It is suggested that arginine may be catabolized in part while in combination as the serum protein.¹²

An influence of the quantity and quality of the dietary protein on the arginase content of the liver has been demonstrated (112). When white rats were fed high-protein diets, the arginase content per unit weight of the liver was increased. Takehara observed a high concentration of liver arginase in fasted animals (113). The high arginase content more rapidly returned to normal when the fasted animals were fed a carbohydrate diet than when they received a fat-protein diet. Hepatic poisons (chloroform, phosphorus) lowered liver arginase content. When the hepatic function was thus depressed, the arginase activity of the kidney increased and arginase could be demonstrated in the spleen (113, 114). The difference in the activities of histidase and arginase preparations strongly indicates that these two enzymes are distinct (115).

The effects of *l*(+)-arginine monohydrochloride (116) and *l*(+)-lysine monohydrochloride (117) on the metabolism of the dog have been compared with those of glycine. Both amino acids were metabolized more slowly than glycine. Lysine exerted no specific

¹² In a note which appeared too late to permit detailed discussion here, Schmidt and co-workers (111) have suggested the possibility of metabolic changes in the free groups of amino acids in protein combination before hydrolysis of the peptide linkages.

dynamic action, while arginine exhibited a specific dynamic action, which, calculated on the basis of the amount administered, was less than that of glycine but which, when calculated on the basis of the calories per millimol deaminized, was slightly higher than that of glycine. Both the basic amino acids were excreted to some extent unchanged depending upon the dosage and the manner of administration. It was notable that despite the excretion of arginine, ornithine could not be detected in the urine in significant amounts.

The biological behavior of canavanine and desaminocanavanine has been studied (118). After the administration of canavanine to a dog, a small amount of desaminocanavanine was isolated from the urine. Both compounds were somewhat toxic to the dog in doses of 200 to 400 mg. per kilo.

THE FORMATION AND DESTRUCTION OF AMINES

A number of papers have appeared which deal with the formation and decomposition of amines derived from the amino acids. Werle & Krautzun (119) have demonstrated the presence of histidine carboxylase in various animal tissues, notably in kidney. The enzyme is not entirely specific for histidine, as tyrosine and tryptophane are also decarboxylated, although proline, alanine, and leucine are not attacked (120).

Holtz, Heise & Spreyer have discussed the relation of oxygen to the activities of tyraminase and histaminase (121). Bhagvat, Blaschko & Richter (122) have studied the distribution of amine oxidase in the animal body. This enzyme, present in most of the mammalian tissues, oxidizes a number of aliphatic and aromatic amines including tyramine and epinephrine (123, 124). It alone is responsible for the variously described epinephrine oxidase and tyraminase activities reported by other workers. Amine oxidase, however, has no action upon histamine or other compounds which contain more than one basic group. The latter type of amine is destroyed in the animal body by a diamino oxidase (histaminase) which has been studied in detail by Zeller (125, 126, 127). The enzyme, prepared from kidney, oxidatively deaminates those compounds which contain two strongly basic groups. Typical substrates are histamine, putrescine, cadaverine, and ethylenediamine. Amine oxidase and diamine oxidase are, therefore, separate enzymes and neither is identical with *D*-amino acid oxidase.

Holtz, Heise & Lüdtke (128) have described an enzyme, present

in guinea pig kidney, which forms hydroxytyramine from *l*-dihydroxyphenylalanine. The enzyme is distinct from amine oxidase and diamine oxidase and is probably not identical with *l*-amino acid oxidase (129).

Loeper and his associates have published a number of papers dealing with the concentration of tyramine in the blood of patients in normal and diseased conditions (130, 131, 132). Normal blood contains between 0.15 and 0.30 mg. per cent of tyramine and the concentration in normal bile is about ten times this value (133). In cases of hepatic and renal disorders, the increases in the tyramine content of the blood and bile paralleled, to a certain extent, the severity of the disease. In severe cirrhosis, blood values as high as 1.1 mg. per cent are recorded (134, 135). The authors believe that both the tyramine and histamine of the blood are formed chiefly by intestinal putrefaction and that the absorbed amines are destroyed in the liver and the kidney. Aminemia results when these organs are not able to carry out their proper function of detoxication.

Ackermann and his associates have isolated histamine from liver and have demonstrated its presence in normal urine (136, 137). Ackermann has reviewed the chemistry and biology of histamine (138) and has suggested a mechanism for histamine action in anaphylactic shock (139). In this connection the quantitative determinations of histamine in anaphylaxis, reported by Code, are of particular interest. During severe shock in dogs and guinea pigs the histamine content of the blood is increased from two to thirteen times the normal value. The increase is not associated with oxygen lack but the histamine is probably responsible for the sudden fall in blood pressure observed in the dogs (140). During anaphylactic shock in horses and calves no increases in histamine could be demonstrated; in most instances a diminution of the histamine in the blood was observed (141). The authors offer no explanation of this interesting species difference in anaphylaxis.

THE POLYPEPTIDE CONTENT OF SERUM

Godfried (142) has examined the existing methods for the determination of the polypeptide nitrogen of blood serum. The higher nitrogen content of the trichloroacetic acid as compared with the phosphotungstic acid filtrate of serum was shown by enzymatic methods to be due almost entirely to peptide nitrogen. The results of a

number of determinations indicated that the polypeptide nitrogen of serum is not more than 7 mg. per cent. In diseases of the liver and kidney and in other disorders where there is an increased parenteral destruction of protein (severe diabetes, leukemia, suppuration), there is usually a marked hyperpolypeptidemia. Cristol & Fourcade (143) have made similar determinations and have attempted a further fractionation of the trichloroacetic filtrate by the use of permutit.

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DEPARTMENT OF BIOLOGICAL CHEMISTRY
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CLINICAL APPLICATIONS OF BIOCHEMISTRY

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The title of this review has been interpreted as covering applications of chemical laboratory procedures to the diagnosis, prognosis, and treatment of disease. Since the introduction of relatively simple chemical methods of blood analysis twenty-five years ago, these methods have played a constantly increasing role in the laboratory and clinic. The reason for this is simple. The blood transports foods to the tissues and waste products to organs of excretion. Abnormalities in metabolism and excretion, and inability to maintain osmotic and acid-base relationships are reflected in the composition of the blood. Although most of the fundamental facts regarding the chemical composition of the blood have already been established, the work in this field is still expanding in the research laboratory and in the diagnostic clinic. For this reason much of the space allotted to this review is devoted to the blood.

Many of the biochemical determinations which have found clinical application have recently been reconsidered from the point of view of developing more accurate methods; of more firmly establishing the normal range of variation; and of determining clinically significant variations from the normal. One is impressed with a decided trend away from the use of whole blood (forsaking "economy" and "convenience") in the various procedures and toward the employment of serum or plasma (preferably separated from blood drawn under oil and without stasis). Such a change is stressed particularly for those determinations wherein the constituent to be determined is unequally distributed between the plasma and the erythrocytes. In considering the various topics of this review, it seemed desirable to discuss the normal range of variation before dealing with the changes observed in clinical conditions. The literature to be cited will cover chiefly the period dating from that of the previous review under the same title (Peters, J. P., Robbins, C. L., and Laviates, P. H.).¹ Since the following subjects, Acid-Base Metabolism (Sendroy, Jr., J.),² and Min-

¹ *Ann. Rev. Biochem.*, 5, 295 (1936).

² *Ibid.*, 7, 231 (1938).

eral Metabolism, Calcium, Magnesium, and Phosphorus (Greenberg, D. M.),³ have recently been considered in these Reviews, they will be omitted here owing to limitation of space. The acid-base balance, mineral elements, and other constituents have also been discussed by us (101) in a more general review.

BLOOD PROTEINS

Hemoglobin.—In the past few years there has accumulated a large amount of data on the hemoglobin concentration of human blood in all parts of the world, a review of which was recently presented by Myers & Eddy. According to these authors it appears that normal adults in good nutrition, not residing at excessive altitudes above the sea level, show essentially the same concentrations of hemoglobin in the blood, without difference due to race or geographic location. The only difference is that due to sex which has long been recognized. The mean of the hemoglobin values reported for strictly normal individuals is 15.8 gm. for the adult male and 13.8 gm. for the adult female per 100 cc. of blood. Andresen & Mugrage reported the following higher mean values, obtained by means of the oxygen capacity method, for normal males and females residing at an elevation of approximately five thousand feet (Denver), namely, 16.58 and 14.45 gm., respectively. McCarthy & Van Slyke found considerably less diurnal variation of the hemoglobin in a series of normal men than had been reported previously. The greatest variation between the highest and the lowest values for one day was 2.3 volumes per cent carbon monoxide capacity, or eleven per cent of the mean value. The average range was 1.3 volumes per cent, or 6.4 per cent of the mean. No uniform direction of change during the day was encountered, however, usually the hemoglobin content was lower in the evening than in the morning. Miller has confirmed the long recognized fact that the hemoglobin concentration slowly declines with age, and noted that this decline is proportional to the decrease in red blood cells. In 160 old men the hemoglobin content declined from 15.3 gm. per 100 cc. at ages 60 to 64 years to 14.1 gm. at ages 94 to 104 years. Guest, Brown & Wing recently reinvestigated the changes of the blood hemoglobin in infancy and childhood in a large series of individuals. Considerable variation between individuals was encountered, however, the mean values showed the following. The mean hemoglobin content of cord blood was 17.9 gm. per 100 cc. and during the first few days of life (one to ten days)

³ *Ann. Rev. Biochem.*, 8, 269 (1937).

the mean value was appreciably higher, namely, 19.0 gm. That this change was not due entirely to a concentration of the blood alone was indicated by the evidence in the blood of new types of cells which were smaller and tended to have a higher concentration of hemoglobin. The hemoglobin content of the blood then fell rapidly so that in the average age groups of 2 months to 4½ years the mean values ranged from 11.0 to 12.8 gm. In children a round number value of 12.0 gm. per 100 cc. is taken as normal (99).

Methemoglobin (sulfhemoglobin).—The cyanosis which almost invariably is experienced following the administration of therapeutic amounts of sulfanilamide has aroused interest in the possible presence of abnormal heme pigments in blood. Such work has precipitated considerable controversy as to whether the darkening of the blood, which is limited to the red cells, is due to a change in the hemoglobin or is due to a staining of the red cells with some product formed during the metabolism of sulfanilamide. That the blood of patients with cyanosis following sulfanilamide treatment may contain methemoglobin has been shown by a number of workers (29, 37, 65, 67, 87, 106, 110, 136, 137) and the same is true in the case of sulfhemoglobin (28, 29, 37, 65, 106). That the blood in some cases of cyanosis following sulfanilamide does not contain abnormal heme pigments or that there is a lack of correlation between the concentration of such pigments and the intensity of cyanosis has been emphasized by a number of workers (23, 37, 87, 110). Consequently, it has been suggested (37, 87, 102, 110) that the dark color of the blood and the cyanosis of the patient are due to the presence of colored derivatives of the sulfanilamide itself. Wendel (136) and Wendel, Wendel & Cox (137) strongly opposed this view and concluded from their observations on nine patients who were cyanotic that the abnormal color of the blood samples was for the most part due to methemoglobin. Hartmann, Perley & Barnett found a marked individual variation in both the rate at which and the degree to which methemoglobin accumulates. These authors found that cyanosis developed in a great majority of the patients receiving 0.1 gm. or more of sulfanilamide per kg. per twenty-four hours and were able to demonstrate the presence of methemoglobin in every case of cyanosis. Wendel (136) found that it was not uncommon to have the functionally active blood pigment reduced by fifteen to thirty per cent. Further, Wendel (135) reported the presence of methemoglobin in every blood specimen which contained more than 4 mg. of sulfanilamide per 100 cc. Hartmann, Perley & Barnett

and Wendel (136) observed that intravenous injection of methylene blue causes a disappearance of cyanosis and a reduction of methemoglobin, the methemoglobin being replaced by an equivalent amount of hemoglobin. Harris & Michel suggested that an active substance is normally produced in the course of sulfanilamide metabolism which causes the production of methemoglobin or sulfhemoglobin. Harris (64) in experiments *in vitro* demonstrated that sulfanilamide cannot function as an oxidizing agent on hemoglobin. However, upon interaction of certain tissues with sulfanilamide, it was found that an oxidizing agent is formed which can cause the production of methemoglobin.

Serum proteins.—Robinson, Price & Hogden (117) found, in studying the determination of the "albumin" and "globulin" fractions of blood serum by precipitation of the globulin in 1.50 *M* solution of sodium sulfate at 37° (Howe micromethod), a variable amount of albumin is adsorbed by the filter paper. It was observed that within rather wide limits the amount of albumin adsorbed is independent of the concentration of albumin, but is dependent upon the type and quantity of paper. Such an adsorption causes an error in the determination of the albumin concentration, which is magnified in calculating the albumin to globulin ratio. This finding undoubtedly explains in part the rather wide variations in the ratio for normal individuals which have been reported by various authors. The above workers found that the error can be avoided by modifying the filtration procedure (117) or by separating the fractions by centrifugation with the angle centrifuge (118). Harris (66) observed an average loss of albumin of 0.05 gm. per 100 cc. with Whatman No. 50 (7 cm. diameter) paper and suggested that this correction be applied where only small samples of serum are available and this type of paper is employed.

In clinical studies Farr & Van Slyke observed, as has been established for adults, a close relation between the presence of edema and the level of the plasma albumin in nephrotic children. These authors found that the edema could be controlled satisfactorily in most instances by simple restriction of salt, together with an adequate diet, when the plasma albumin level was above 1.2 gm. per 100 cc. The latter value is definitely below the generally accepted critical level of 2.5 gm. per 100 cc. for adults. Recent studies of the plasma protein in hepatic disease (18, 43, 132) have reaffirmed previous findings of a lowered albumin concentration. The globulin content tends to be

increased resulting in a decided alteration of the albumin to globulin ratio. The total plasma protein level is variable depending upon the extent of alteration of the albumin and globulin fractions.

The number of publications which have appeared in the last few years reporting instances of hyperproteinemia leads one to the conclusion that this condition is not so rare as was once believed. Williams & Gutman reported on twelve patients with lymphogranuloma inguinale with a total serum protein of over 8.0 gm. per 100 cc. in ten cases. In eleven patients there was a relative and absolute increase in the globulin with a reversal of the albumin to globulin ratio in nine instances. In a later study Gutman & Gutman included fifty observations on 39 patients in whom the total serum protein exceeded 8.0 gm. per 100 cc. in thirty-seven observations, the highest value being 11.4 gm. per 100 cc. with an albumin content of 2.6 gm. The presence of hyperproteinemia in multiple myeloma is now recognized. Feller & Fowler reviewed the literature and tabulated the findings in fifty-two cases of multiple myeloma with hyperproteinemia. This tabulation led to the finding that the albumin content was generally within normal limits, however, the globulin concentration was consistently elevated. The highest total protein concentration recorded was 18.37 gm. per 100 cc. In their own series of ten patients Feller & Fowler found the plasma protein to exceed 9.0 gm. per 100 cc. in three cases. Bing, Mills & Pritchard, Jeghers & Selesnick, and Ulrich also have noted in multiple myeloma hyperproteinemia which was the result of an increased globulin content. Harrell & Fisher reported on eleven cases of Boeck's sarcoid and observed a total serum protein concentration above 8 gm. per 100 cc. in eight patients. A reversal of the albumin to globulin ratio was noted in all patients during the active stage. Other clinical conditions wherein hyperproteinemia has been observed include cirrhosis of the liver (60), various infections (60), and dehydration (78).

NON-PROTEIN NITROGEN CONSTITUENTS OF BLOOD

Urea nitrogen.—Howell has considered the errors encountered in the methods for determining blood urea which employ jack bean meal extracts to hydrolyze the urea. This author presented evidence for the presence in fresh extracts of a labile substrate-enzyme system which produces ammonia. Upon standing this system becomes inactive whereupon a second enzyme, probably arginase, becomes active and also produces ammonia. It was found that the error encountered, which

may be of appreciable magnitude, can be eliminated by the use of a five- to twenty-hour jack bean extract and the adjustment of the pH of the digest to between 6.0 and 6.6 by the use of citrate buffer. Lewis & Alving have determined the level of the blood urea in a large series of normal men ranging from 40 to 101 years of age with the finding that the level increases with age. The equation for the increase of urea in men aged 40 to 89 years was found to be,

$$\text{Urea nitrogen (mg. per 100 cc.)} = 7.56 + (0.112 \times \text{age in years}).$$

A number of factors have been suggested to explain the elevation of blood urea nitrogen following hematemesis or melena (2, 12, 25, 26, 121). These include the digestion and absorption of the blood in the intestine, dehydration, starvation, "toxic" destruction of body protein, and impairment of renal function. Following a single severe, non-fatal hemorrhage from the stomach or duodenum there appears to be an increase in the blood urea nitrogen (up to double its normal value or more) in the course of six to twenty-four hours (2, 26, 121). The value then generally returns to normal in a few days (26, 121) or in some instances may remain somewhat elevated for longer periods (2). A similar response has been demonstrated in humans following intragastric or intrajejunal administration of citrated human blood (121) and ox blood (26) or following the ingestion of a large quantity of lean beef (121). In patients showing continued or recurrent bleeding the urea nitrogen remains high and markedly elevated values may be encountered in cases which by repeated massive bleeding or by operation develop a shock syndrome (2, 12). At present it appears that the most likely answer for the blood urea elevations lies in the digestion and absorption of the blood proteins and in prerenal factors.

Falconer has recently reconsidered the blood chemistry in intestinal obstruction and feels that clinically the changes are secondary to the onset of intoxication. That is, cases of either high or low intestinal obstruction were encountered in which the obstructing lesion had been present for some time without the development of significant alterations in the blood chemistry. A rise in the concentration of blood urea was found to be the most constant change and was associated (except in cases associated with nephritis) with a high urinary output of nitrogen. This was regarded as evidence of an increased endogenous metabolism of protein. The alterations of the concentration of blood urea were found to afford better criteria of the patients' progress than changes in the plasma chloride concentration and a

steady and rapid rise in the blood urea was regarded as of grave prognostic significance.

The elevations of blood urea nitrogen in clinical conditions accompanied by dehydration have been discussed by McCance (88, 89). In some interesting experiments McCance (89) was able to induce salt deficiency in normal humans by salt deprivation and sweating. Among other things, it was found that in such individuals the blood urea level became doubled or more, there was a negative nitrogen balance and the creatinine, sucrose and urea clearance values were definitely depressed. The problem of extrarenal azotemia has been reviewed by Jeghers & Bakst and they discuss the subject on the basis of the following six basic mechanisms: drop in blood pressure, hypochloremia and hyponatremia, dehydration, liver damage, protein catabolism, and local renal disturbance. It is pointed out that the mechanisms are interlinked and often interdependent.

Uric acid.—Studies on the uric acid content of the blood in the past few years have emphasized the fact that one must know, in addition to the method employed, whether the analysis has been made on whole blood, unlaked whole blood or plasma or serum (separated under oil) before comment can be made on the normality or abnormality of a given figure. Blaich & Koch have been able to show, with the aid of uricase, that the results obtained with the direct method of determining uric acid are about one-third too high, i.e., too high by about 1.0 mg. They found the average value for fifty samples of human blood to be 2 mg. per 100 cc. with a range of 1.04 to 3.83 mg. The apparent uric acid for these samples obtained by the direct procedure, without uricase action, ranged from 1.82 to 4.60 mg. with an average of 3 mg. per 100 cc. of blood. The recent studies of Talbott & Coombs and Jacobson (76) have indicated that more reliable information regarding uric acid may be obtained from the analysis of serum, especially if separated under oil. Employing serum thus separated Jacobson found the fasting uric acid in one hundred non-gouty individuals on a mixed diet to range from 1.9 to 6.7 mg. per 100 cc. with a mean of 4.2 mg. and in ninety-seven individuals the value was less than 6.0 mg.

Clinically, the recent studies of blood uric acid have centered around gout, a condition long recognized as one in which the uric acid values are increased. Jacobson found in 177 analyses on 21 cases of gout that the serum uric acid ranged from 5.2 to 14.8 mg., ninety-eight per cent of the values exceeding 6.0 mg. and ninety-four per

cent exceeding 7.0 mg. per 100 cc. He observed some evidence of correlation between the onset of an attack, the severity of the disease and the level of the serum uric acid. Talbott & Coombs (129) in a series of 24 cases of gout found the serum uric acid values to range from 5.7 to 14.2 mg. per 100 cc., the average value being well over 7.0 mg. In another study Talbott & Coombs (128) examined the serum of 68 non-affected members of families of sixteen gouty patients and found that fourteen (slightly over twenty per cent) showed concentrations of uric acid greater than 6.0 mg. They are of the opinion that these findings support the hypothesis that gout is a familial disease and that one manifestation of it, i.e., an elevation of the serum uric acid, may be subject to hereditary transmission. The heredity in gout is also stressed by Hill and is supported by the finding of Jacobson who noted elevated uric acid values in the son of each of two gouty individuals and in the brother of the third, they themselves being free of gout. Price has reported five cases of congestive heart failure in which attacks of gout followed diuresis produced by salyrgan. This author comments that in view of these observations the nature of the diuresis effected by salyrgan in the edematous subject assumes considerable interest, since the "critical" diuresis achieved by the salyrgan closely parallels a "critical" diuresis preceding the ordinary attack of gout, the latter having been pointed out previously by Talbott and his co-workers (129). Several authors (27, 71, 122) have taken the view that gout is not to be regarded as an uncommon and disappearing disease.

Creatinine.—Since Behre & Benedict in 1922 first presented evidence which they believed disproved the presence of creatinine in blood a bitter controversy has raged over this question. Gaebler, who first opposed Benedict, was later in part converted to his point of view. Although Gaebler was able to isolate creatinine from both normal and pathological bloods, he conceded that blood filtrates differed from solutions of pure creatinine in their response to certain physical and chemical reactions, and thus creatinine as such was not present in detectable amounts in normal blood. In affirming their original position, Benedict & Behre (8, 9) have made the additional observations that the color obtained with blood filtrates and diluted serum ultrafiltrates with 3,5-dinitrobenzoate differs from that of creatinine, and that plasma filtrates of human and other species fail to precipitate under conditions which precipitate added creatinine. Gaebler observed that a part of the apparent creatinine of normal serum ultrafiltrates

simulates creatinine in its precipitation behavior, is not identical with it, but may be related to it. He found that in the case of both normal dog and human blood the apparent creatinine of laked blood ultrafiltrates is not removed by kaolin while added creatinine is removable. Evidence in opposition to the views of Benedict has been presented by a number of workers. Hayman, Johnston & Bender (68) found that when trichloroacetic acid (instead of picric acid) was used to precipitate the proteins of serum or plasma, the filtrate differed little in its reactions from pure creatinine. From a study of plasma ultrafiltrates Danielson (32) arrived at much the same conclusion. The problem has been approached in a different way by Miller & Dubos. With the aid of a specific bacterial enzyme they have found that creatinine constitutes eighty to one hundred per cent of the chromogenic material in normal serum or plasma, although in uremia there may be large amounts of non-creatinine, chromogenic material, which in several patients seemed to parallel the severity of uremic symptoms. Gaebler (44) and Gaebler & Abbott (45) have shown that most of the apparent creatinine of ultrafiltrates of normal blood can be precipitated with picric acid and rubidium, while Goudsmit has compared the apparent creatinine content of renal venous blood and of arterial blood and found the former consistently lower, thus supporting the view that the apparent creatinine of blood is the precursor of urinary creatinine. The outcome of the controversy would appear to possess far greater theoretical than clinical importance, since it makes little practical difference whether urinary creatinine is derived from creatinine or apparent creatinine in the blood.

Guanidines.—Since Major & Weber in 1927 utilized and improved the Marston color reagent for the estimation of guanidine, and reported an increased blood concentration of guanidine-like substances in hypertension, the guanidines have continued to command interest. In their earliest publications Major & Weber emphasized the elevation of "blood guanidine" in arterial hypertension. They reported that although some patients with "essential hypertension" showed normal values for "blood guanidine," the majority of patients showed some increase, while patients with nephritis and nitrogen retention showed a marked increase in "blood guanidine." That the "blood guanidines" are elevated in nitrogen retention is generally conceded, but there has been less confirmation of the statement regarding essential hypertension.

Andes, Linegar & Myers studied fifty-five cases with nitrogen re-

tension and twelve with hypertension but without nitrogen retention. In general the blood guanidines were found to follow the azotemia, and appeared to bear no direct relation to the hypertension. All but one of the cases of hypertension without nitrogen retention showed normal values. Child observed in dogs that the guanidine bases do not rise in the blood stream following ligation of one renal artery unless nitrogen retention appears. Helmer & Page observed that guanidine-like substances gave physiological properties similar to those that might be expected of an effector substance liberated from the kidneys when the renal arteries are constricted by Goldblatt's clamp. Despite this no significant increase was found in the whole blood of dogs made severely hypertensive by this method when no rise in the urea nitrogen occurred.

Recently Major (84) has stated that continued investigation has convinced him that his first statement on this subject in 1927 is still valid: "the blood of certain patients suffering from arterial hypertension contains something which is present in greater amounts than in normal blood." With the Major and Weber method, Major finds the normal "blood guanidine" does not exceed 0.2 mg. per 100 cc. In a study of 800 cases, 625 of whom showed nonprotein nitrogen values of 40 mg. or less, 200 gave an increased content of guanidine. In a more recent study on dogs Major, Weber & Rumold conclude that the increase in "blood guanidine" in experimental renal insufficiency occurs both with and without accompanying hypertension, and the compound which they have designated as guanidine is apparently a guanidine derivative of an anhydride type and may be glycohydrazide.

Increased guanidine values have been observed by Andes, Andes & Myers in severe toxemias of pregnancy. The hyperguanidinemia was found to continue as long as the toxic condition existed. Markedly increased guanidine values were always found with eclamptic convulsions. In opposition to the theories advanced by Noel Paton and his school some years ago, Andes & Myers found essentially normal values for blood guanidine in eight cases of parathyroid tetany (following subtotal thyroidectomy) and in one case of idiopathic tetany.

BLOOD LIPIDS INCLUDING CHOLESTEROL

In the previous review Sperry⁴ discussed the cholesterol content of the blood serum in health and emphasized its wide variability.

⁴ *Ann. Rev. Biochem.*, **8**, 231 (1939).

The values range from 147 to 322 mg. per 100 cc. and Sperry stated, "In view of the wide variation in the cholesterol concentration of the serum or plasma in health apparent deviations from the normal in the presence of disease must be interpreted with caution." It is evident that with a given method of determination adequate controls must be obtained.

Recent studies (14, 15, 17, 20, 54, 92, 116) lend support to the earlier findings that the plasma cholesterol determination (or total lipid) may be of considerable clinical value in thyroid disease. As a general rule elevated values are encountered in hypothyroidism and lowered values in hyperthyroidism. The determination may not only be of value in diagnosis but also as an aid in therapy.

From their intensive study Epstein & Greenspan found that repeated determinations of free and ester blood cholesterol enabled them to improve their accuracy in the diagnosis and prognosis of hepatic and biliary diseases. These authors observed that in obstructive jaundice hypercholesterolemia is usually encountered, affecting both the free and ester fractions, which parallels the degree of hyperbilirubinemia. In jaundice occurring in acute degeneration of the liver, blood cholesterol did not rise with the bilirubin, but usually remained low or subnormal. The cholesterol ester was usually lowered in acute degeneration of the liver and mirrored the severity of the damage. In rapidly fatal cases the ester was low or even absent throughout the course of the disease. The more recent observations of others (16, 123, 138) tend to confirm the above findings. Boyd & Connell (16) point out that the decrease of ester to total cholesterol in parenchymatous hepatic disease is in reality part of a general lipopenia.

In the chronic active stage of chronic glomerular nephritis, Page, Kirk & Van Slyke (104) observed a tendency to lipemia, with plasma lipids near or above the upper limits of normal. They found further that as the disease passes into the terminal stage, the lipemia is likely to decrease and before exitus the plasma lipid content may fall below normal. The individual lipid constituents, free cholesterol, cholesterol esters, phosphatides, and the neutral fat fraction were found to rise and fall together. In confirmation Herbert (70) also found lipemia associated with renal edema, all the lipid fractions being increased. Page & Farr found that the plasma lipids in patients with nephrosis or the nephrotic stage of hemorrhagic nephritis with hyperlipemia are not regularly influenced by the amount of fat in the diet or by the administration of thyroid. Thyroid administration sufficient to in-

crease markedly the basal metabolic rate had no obvious effect on the plasma lipids. The latter finding is in striking contrast to the fall in lipids parallel with the rise in basal metabolic rate observed in patients with myxedema receiving thyroid (54). The more recent reports dealing with the cholesterol content of the blood in essential hypertension and arteriosclerosis are not entirely uniform. Page, Kirk & Van Slyke (105) found values for the concentration of total lipids and the various lipid fractions within the normal range in uncomplicated essential hypertension. Elliot & Nuzum (35) also failed to find a hypercholesterolemia in similar cases. Davis, Stern & Lesnick (33) reported that the average values for cholesterol and other lipid fractions were higher in the blood of patients with angina pectoris and arteriosclerosis than in normals. Poindexter & Bruger (109) reported a significant elevation of the plasma cholesterol in patients with arteriosclerotic heart disease or hypertensive heart disease manifesting some evidence of arteriosclerosis.

The blood lipids have been studied by Man & Gildea (86) in malnutrition, and in most cases were found reduced. These authors suggested that a part of the hypoproteinemia and hypolipemia associated with debilitating diseases such as nephritis, tuberculosis and possibly hyperthyroidism may be attributed to the state of malnutrition.

The total plasma lipid content and lipid fractions in xanthomatous diseases have been studied recently by a number of workers (21, 70, 98, 130, 134). The reader is referred especially to the paper by Thannhauser & Magendantz for a classification and review of the xanthomatoses.

SERUM BILIRUBIN

The excretion of bilirubin by the liver has frequently been compared with the excretion of urea by the kidney. In the absence of biliary obstruction or hemolysis it is generally assumed that increased quantities of bilirubin in the blood are due to hepatic disease. The value of the level of serum bilirubin as a measure of hepatic function has been discussed by Snell & Magath. That the activity of the external secretion of the pancreas may be altered in diabetes has been noted (100). Rabinowitch has reported a high incidence of hyperbilirubinemia and excess quantities of urobilinogen in the urine in diabetes, thus affording proof of the occurrence of liver disease in many diabetics. Comparing the bilirubin content of the serum of 85 cases of pernicious anemia with the same number of normals Mills & Maw-

son found values of 0.98 ± 0.06 mg. and 0.31 ± 0.02 mg. per 100 cc. respectively. In 53 cases of pernicious anemia controlled by liver therapy the serum bilirubin was 0.31 ± 0.03 mg. per 100 cc., demonstrating that the specific therapy reduced the raised values to normal.

BLOOD IODINE

A number of investigators have considered the metabolism of iodine in normal persons and in patients with goiter. The range of the blood iodine in individuals not on iodine medication which has been taken as normal in these studies appears to vary quite appreciably. Thus, Stevens reported for normal individuals an average of 4.8 micrograms per 100 cc. of blood with variations between 3.6 and 6.9 micrograms. Bauman & Metzger found in ten normal males an average value of 3.5 (range 3.0 to 4.4) and in ten normal females an average of 2.6 (range 2.3 to 3.0) micrograms per 100 cc. Puppel & Curtis (113) considered 2.2 micrograms per 100 cc. as a low normal and a value of 6.9 micrograms per 100 cc. as elevated. Fashena studied the blood iodine in one hundred normal children ranging from birth to thirteen years, and found in 79 subjects from the age of two days to thirteen years that the blood iodines varied from 3.0 to 12.0 micrograms per 100 cc. with an average of 6.6 ± 0.15 micrograms. In 21 infants under twenty-four hours of age the values were found to be significantly lower, ranging from 1.0 to 11.0 micrograms per 100 cc. with an average of 4.7 ± 0.33 micrograms. McCullagh & McCullagh reported that the blood iodine in normals and in patients who had diseases not associated with the thyroid gland ranged from approximately 8 to 12 micrograms per 100 cc. Puppel & Curtis (112) considered values of 7.9 and 13.4 micrograms per 100 cc. in the normal range while Perkin & Lahey took 10 micrograms of iodine per 100 cc. of whole blood as the upper limit of normal.

A number of workers have considered the clinical significance of blood iodine determinations, particularly in reference to thyroid disorders. Fashena, admittedly studying only a relatively few cases with thyroid dysfunction, found that although the blood iodine values were suggestively low in hypothyroidism and high in hyperthyroidism, they frequently fell within normal limits. Consequently it was concluded that although useful in confirming a clinical impression the determination has very little absolute diagnostic significance. McCullagh & McCullagh, while questioning the value of the blood iodine in the diag-

nosis of hypothyroidism, emphasize its value in the differential diagnosis of hyperthyroidism. Puppel & Curtis (112) in their metabolic study reported a lowered blood iodine value in a case of hypothyroidism and elevated values in two cases of exophthalmic goiter. In a later study these authors (31) reported from a study of 24 patients with exophthalmic goiter that the blood iodine was greatly increased in 21 patients, slightly increased in one and normal in two. Further, in nine patients with toxic nodular goiter, the blood iodine was increased in eight and normal in one. Perkin & Lahey called attention to their previous findings of an elevated blood iodine level in seventy per cent of cases of clinical hyperthyroidism, the remaining thirty per cent being within the normal range. The latter authors questioned whether a relation exists between the blood iodine level and the duration of the syndrome of hyperthyroidism. Therefore they correlated the level of the blood iodine with the duration of the symptoms in 305 cases of exophthalmic goiter. It was found that the iodine level of the blood was elevated in the majority (eighty-six per cent) of cases in which symptoms had been present from one to nine months. The iodine level tended to fall within the normal range in those patients in which the symptoms had been present for one year or longer. An absence of a proportional relation between the degree of elevation of the iodine content and basal metabolic rate was clearly demonstrated. Perkin & Hurxthal fractionated the iodine of blood by alcohol precipitation in patients with nontoxic goiter, exophthalmic goiter, and primary myxedema. The iodine recovered in the alcohol insoluble fraction (termed organic blood iodine and representing somewhat more than sixty per cent of the total blood iodine at normal levels) was found to be decreased in the patients with primary myxedema. The lowered blood iodine noted in myxedema appeared, therefore, to be entirely the result of a fall of the organic blood iodine and the authors suggested that its determination might prove of great value in the diagnosis of thyroid insufficiency. An elevated blood iodine was noted in cases of exophthalmic goiter who had received no iodine and this was the result of a relatively greater increase in organic iodine than inorganic iodine. Similar cases receiving iodine medication were found to have relatively less organic blood iodine. In cases of nontoxic goiter receiving iodine medication the blood iodine was also found to be increased due to an increase of both the organic and inorganic fractions. No correlation between organic blood iodine and basal metabolic rate was found. It is of interest in this connection that

Trevorrow presented evidence which indicates that the iodine in the blood of animals on an iodine-poor diet is not in combination with protein and not more than twenty per cent is in inorganic form. It was concluded that the greater part of the blood iodine possesses properties similar to those of thyroxine and diiodotyrosine, and a portion of this is not diiodotyrosine but is like thyroxine in its solubility.

Curtis & Puppel (31, 112, 113) have stressed the excessive excretion of iodine in certain thyroid disorders.

BLOOD VOLUME

The measurement of the blood volume with the aid of the colloidal blue dye T-1824 has recently been reinvestigated (47, 51, 58). Gibson & Evans (48) found the blood volume in normal persons to vary within wide limits, however, it was concluded that the relationship to height or surface area offers a useful basis for the estimation of normal values in clinical investigation. The average value for the total blood volume for males and females (all ages) was found to be 77.7 and 66.1 cc. per kilo body weight, respectively, and the average plasma volume to be 43.1 and 41.5 cc. per kilo body weight, respectively. It was observed that with increasing age there is a decline in the blood volume comparable to decreases in basal metabolic rates and vital capacities.

Clinical studies have been made in congestive heart failure by Gibson & Evans (49) and it was observed that the change from the compensated to the decompensated state was accompanied by a progressive increase in the volume of plasma and red cells. This increase was shared to a slightly less extent by the plasma than by the corpuscles, resulting in a slight concentration of the blood. These same authors (50) found in twenty-five cases of hyperthyroidism that the total blood volume was above normal on the average of 5.45 per cent, while in seven cases of myxedema it was 15.5 per cent below normal. Gibson (46) observed a hydremic hypovolemia in the severe stages of pernicious anemia. Thus, in a group of ten cases the average plasma volume was thirty per cent above, circulating red blood cell volume sixty-eight per cent below, total blood volume fourteen per cent below, and total hemoglobin seventy per cent below normal, at a red cell level of 1.5 million. Harris and Gibson (63) feel their observations clearly indicate that in subacute or chronic glomerular nephritis with or without renal edema or renal insufficiency, but without congestive heart failure, the plasma volume is above, and the cir-

culating red cell and total blood volume are below the values for normal individuals. Furthermore, the diminution in circulating red cell and total blood volume tends to be more severe in subacute glomerular nephritis with renal edema (nephrosis syndrome) than in glomerular nephritis without edema. Their observations led to the conclusion that in Bright's disease the plasma volume tends to vary directly with the serum albumin concentration and blood nonprotein nitrogen concentration and indirectly with the degree of anemia present. In chronic nephritis complicated by heart failure, the plasma, circulating red cell, and total blood volume are definitely above normal levels found at comparable levels of anemia in patients with chronic nephritis but without congestive heart failure. In a very recent study Gibson, Harris & Swigert emphasized the striking relationship between the severity of anemia and the level of the plasma, circulating red cell, and total blood volume and concluded that in chronic anemias, regardless of etiology, plasma volume is above and circulating red cell and total blood volume are below normal. It was concluded further that for clinical purposes the hematocrit level is a better criterion of the degree of deficit in circulating red cell volume than the red cell count or hemoglobin determination. The above authors observed an increased total blood volume in polycythemia vera, due entirely to a great increase in circulating red cells. Haden also found the blood volume constantly increased in polycythemia vera with a plasma volume seldom above normal (congo red used).

In artificial fever Gibson & Kopp noted a considerable loss of plasma volume in cases where fluid was not given and state that for each individual there is a definite limit beyond which further loss of fluid from the blood stream cannot be tolerated. Gilligan, Altschule & Volk, who studied the effect of fluids administered intravenously in man, found the plasma and blood volumes appreciably increased up to approximately two hours after injections of 1000 cc. of 0.85 per cent saline or of five per cent glucose in 0.85 per cent saline at rates of 30 cc. per minute or greater. It is indicated that after intravenous injection of fluids, the forces which govern the escape of fluid from the circulation normally act to resist increases in blood volume of over approximately twenty per cent.

TESTS OF HEPATIC FUNCTION

It is not possible to review the numerous publications on tests of hepatic function and consequently only certain conclusions regarding

the usefulness of some of the tests will be considered. Attention should be called to the review by Snell & Magath of a series of tests for liver function which find most frequent use.

Hippuric acid test.—Recent observations on the hippuric acid test in cases of disease of the liver (80, 114, 124, 125, 127, 138, 140, 141) have led to its recommendation for general use, particularly in evaluating prognosis and surgical risk. In general, it appears that in patients who are not jaundiced the results of the hippuric acid test closely parallel those of dye tests (bromsulfalein or rose bengal) and of more importance, it apparently yields helpful information in jaundiced patients wherein the dye tests fail.

Quick (114), from a study of the test in 158 patients with hepatic or biliary disease, found a low output of hippuric acid occurs in cases of catarrhal jaundice and in various forms of hepatitis and usually in cases of a malignant process with metastasis to the liver, syphilitic cirrhosis and atrophic and hypertrophic cirrhosis. Reaction to the test was found normal in cases of cholecystitis, cholelithiasis and biliary obstruction due to stones in the common duct if the condition was of short duration. Quick felt that the test was promising as a means for estimating hepatic insufficiency and as an aid in the differential diagnosis, especially in distinguishing jaundice of hepatitis and the jaundice arising due to a stone in the common duct. Yardumian & Rosenthal found the test valuable to differentiate intrahepatic and extrahepatic jaundice but this is denied by Snell & Plunkett and others (138). Quick found, in agreement with others, that repeated determinations are useful in following the course of disease of the liver and in determining the effectiveness of therapy. Snell & Plunkett studied 38 patients and concluded that their results seem to indicate that the rate of synthesis of hippuric acid is a reasonably accurate and satisfactory test for the determination of parenchymatous hepatic damage, particularly in the "surgical" types of jaundice. They believe that in cases in which the elimination of hippuric acid is reduced by more than fifty per cent, severe damage of the hepatic parenchyma can be assumed to exist, and that surgical procedures will entail a considerably increased risk. These authors found in general an agreement between the degree of bilirubinemia and the rate of hippuric acid synthesis. Kohlstaedt & Helmer concluded from their study of 77 patients that there may be a marked reduction in the detoxifying ability of the liver before any of the clinical signs of hepatic disease can be detected. These authors pointed out, as had been noted earlier, that

the rate of excretion of hippuric acid depends on the kidney function as well as on the rate of synthesis of glycine and its conjugation. Consequently it was suggested that the simultaneous determination of the urea clearance increases the value of the test. They stated that this combined liver and kidney function test offers an added safeguard in the preoperative study of surgical cases, because postoperative complications which may result in the so-called "liver death" are most likely to occur in those cases in which the hepatic detoxifying mechanism or the renal function is impaired. The most recent reports (127, 138, 140) serve to emphasize the frequency of a reduced hippuric acid elimination in patients with liver damage.

In interpreting the results obtained with this test account must be taken of possible renal injury (80), congestive heart failure (141), dehydration and malnutrition (124) which depress hippuric acid excretion. To obviate possible incomplete absorption of the benzoate, Lipscutz (82) has modified the usual test by administering the drug intravenously.

Takata-Ara test.—The Takata-Ara test, which supposedly is of value in identifying cirrhosis of the liver, has been critically examined from this point of view in several recent publications (13, 22, 73, 75, 79, 83). One gains the impression that a positive reaction does offer some confirmatory evidence in the diagnosis of cirrhosis of the liver while a negative test in a suspected case would lead one to question the diagnosis.

From a review of the reports in the literature Kirk found the test to be positive in 315 out of 375 cases of cirrhosis of the liver. In his own experience the test was positive in 15 out of 21 cases of clinical cirrhosis. It was also found to be positive in other patients with and without evidence of liver damage, particularly when the serum globulin was elevated, and Kirk came to the conclusion that the test is not diagnostic of cirrhosis of the liver. Magath came to the conclusion that the test will be positive in slightly more than half of the cases in which there is parenchymatous hepatic damage when the injury has reached a moderately severe stage and, therefore, is not specific for any one disease of the liver. It was found further, that whereas the test is frequently found positive in advanced cirrhosis, in the earlier stages many negative tests are obtained. As a result of their study Bowman & Brady came to the conclusion that the test is not significant enough to be of value in the clinic as an additional laboratory procedure. Hořejši found the test positive in eighty-three per cent of

the cases with liver cirrhosis and whereas it was frequently found positive in other conditions he believed that the test is useful since a strongly positive reaction supports the diagnosis of liver cirrhosis. Chasnoff & Solomon found the test positive in cases of advanced liver cirrhosis and in most cases of malignancy involving the liver. In other patients without liver damage a positive reaction was not infrequently encountered. These authors concluded that the test cannot be regarded as specific for cirrhosis, but a negative reaction in any suspected case would tend to cast doubt on the diagnosis.

Phosphatase.—Considerable confusion exists in the literature regarding the phosphatase activity of the blood in diseased conditions for the reason that a variety of techniques have been employed, there has been no uniformity of expressing phosphatase units, and in some instances authors employing the same procedure have reported appreciably different ranges for the normal. The normal range as obtained by the various methods as well as the definitions of phosphatase units have recently been discussed by Roe & Whitmore.

Clinical studies of the phosphatase activity of the blood of patients with jaundice which have appeared in the last few years have continued the controversy as to its value as a means of differentiating between obstructive and non-obstructive jaundice. Thus Rothman, Meranze & Meranze (120), in support of some earlier work, concluded that the phosphatase determination is of considerable value. By employing a slight modification of Roberts' method these authors observed that most cases of obstructive jaundice had values greater than ten units while in the non-obstructive cases the values were found to be ten units or less. Although their experience was admittedly limited Snell & Magath reported agreement with the above. Flood, Gutman & Gutman found the phosphatase activity of the serum to be increased in every case of jaundice due to obstruction of the common bile duct. These authors took the view that values for phosphatase activity which are not definitely elevated tend to rule out jaundice due to obstruction of the common bile duct, and consequently the method is useful in the differential diagnosis of jaundice. Shay & Fieman, who discussed the significance of superimposed disturbances in the two groups of jaundice, came to the conclusion that in the pure obstructive group the phosphatase value is informative. In contrast to the above, however, Cantarow & Nelson, Morris & Peden, and Giordano, Wilhelm & Prestrud reported a rather wide overlapping of the phosphatase values in the two groups. Consequently, in support

of other earlier workers, it was concluded that the procedure is of little or no value in the differential diagnosis of obstructive jaundice.

Plasma phosphatase in disease, especially in reference to lesions of the bones, has been reviewed recently by Morris & Peden and Roe & Whitmore.

TESTS OF DIGESTIVE FUNCTION

Following the successful purification and crystallization of secretin, Ågren, Lagerlof & Berglund used this material as a stimulant for pancreatic secretion. Duodenal contents were collected and analyzed for enzyme activity and for bicarbonate concentration. The secretin test showed pathologic deviations in cases of acute pancreatitis, carcinoma of the pancreas, and stone in the pancreatic duct. Several cases which had been tentatively diagnosed as chronic pancreatitis presented normal findings. The fact that secretin is a potent stimulus of pancreatic secretin may in some part have caused an apparently normal response even from a diseased pancreas. Myers, Free & Beams have suggested improved chemical methods for the estimation of enzyme activities in duodenal contents. In order to simplify the clinical interpretation of the results of such enzyme analyses they have further proposed that enzyme activities be expressed as a percentage of one hundred which is taken as the average normal. A number of cases suffering from miscellaneous gastro-intestinal disorders gave subnormal values suggestive of reduced pancreatic activity although no definite pancreatic disease was noted. Comfort, Parker & Osterberg employing methods in which the normal range of enzyme activities was quite large report no contraction in range in patients with chronic pancreatitis without steatorrhea, whereas in patients with chronic atrophic pancreatitis with steatorrhea a marked diminution in enzyme activity occurred. A differentiation of the steatorrhea of non-tropical sprue from steatorrhea of chronic atrophic pancreatitis could be made from enzyme analyses of duodenal contents since the former condition presented normal figures according to the standards of these investigators. Diamond, Siegel, Gall & Karlen have obtained promising results using secretin stimulation as suggested by Ågren and his colleagues.

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HORMONES

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Because the more definitely biochemical advances in the field of hormones are very nearly limited to those of the steroid type together with the limited space available for reviewing a very extensive literature, I have taken advantage of the editors' liberal policy and have limited this review to the biochemistry of the steroid hormones.

ESTROGENS

Distribution and forms of estrogens.—Although all of the estrogen activity in cow and hog ovaries is considered to be in the free form, only 50 to 75 per cent is free in the horse ovary or its follicular fluid (1). During pregnancy 50 to 75 per cent of the blood estrogens in women are in the free form (2). The best method of hydrolysis of the conjugated forms of estrogens in pregnancy urine is still a problem. One group recommends boiling the pregnancy urine for ten minutes with 15 per cent concentrated hydrochloric added. Longer heating results in serious loss of estrogens (3). Others recommend that the urine from pregnant mares be allowed to stand at pH 0.4 to 0.6 at room temperature for at least four weeks (4). The writer, in studies on urine from normal men and nonpregnant women, has obtained the highest yield of estrogens by boiling the urine previously acidified to pH 1 to 1.2 with hydrochloric acid for fifteen minutes. Longer boiling at this pH also causes destruction of estrogens. The gradual increase in the urinary excretion of estrogens in women to a peak of 80,000 rat units near the termination has been confirmed, but in one case of toxemia of pregnancy, the excretion was subnormal. Treatment of the latter case with estradiol benzoate relieved the symptoms (5). In castrated women, estrogen excretion was increased after dihydrofolliculin benzoate therapy but not after progesterone or testosterone (6). After menopause considerable amounts of estrogen are still excreted by women (7). Although estrogenic activity was found in bull, ram, cat, dog, and boar testicles, only the urine of the boar gave a positive estrogen test (8). Dried sage leaves assayed 6,000 I.U. per kg. (9).

Conjugated forms of estrogens have been synthesized by shaking the estrogen or androgen with methyl acetobromoglucuronate in benzene. The esters were hydrolyzed by barium hydroxide. None of the derivatives were very active estrogens, and all of the androgen derivatives were inactive by direct application on the capon comb. With total doses of 30 to 60 μg .¹ estrogen activity was demonstrated for α -estradiol-17-glucuronic acid, methyl-estrone-triacetyl-glucuronate, and methyl- α -estradiol-3-benzoate-17-triacetyl-glucuronate (10). The presence of estrone sulphate in pregnancy urine has been confirmed (11, 12). It has also been prepared by allowing chlorosulphuric acid to act with estrone in a mixture of pyridine and chloroform at 20° C. for twenty-four hours. The estrone sulphate possesses only 2 per cent of the potency of its estrone content (12).

Estradiol has been separated as the digitonide from the nonketonic fraction from human pregnancy urine (13).

Further evidence has been presented indicating the possible presence of another type of estrogen in hog ovaries. The evidence involves the comparative results obtained by assaying extracts from follicular fluid and from an ovarian tissue residue left after dissecting the corpora lutea and aspirating the follicular fluid on spayed rats and immature mice respectively. Mixtures of pure estrogens, alone or with pure or impure progesterone, were not assayed (14).

Estrogen assay methods.—The immature rat uterine weight method has been modified and studied more extensively (15). The uterine response to increasing doses of estrone and estradiol is very similar, but for estriol the curve reaches a plateau at relatively low levels. By this method estradiol is about twenty times as potent as estrone (16).

The color reactions of bile acids, sterols, and estrogens with vitamin C, furfural, and sulphuric acid have been studied quite intensively, (17) a new color reaction has been applied quantitatively on estriol, (18) and the Kober method for total estrogens has been modified with the use of the Evelyn colorimeter (19). The modified Kober method covers a range equivalent to 10 to 60 μg . of estrone.

Further advances in the synthesis of estrogens are relatively limited. Treatment of 1-vinyl-6-methoxy-3,4-dihydronaphthalene with 1-methyl-cyclopentene-2,3-dione yields a complex which with hydrogen bromide yields a dehydroestrone, $\text{C}_{18}\text{H}_{20}\text{O}_2$, and the latter by catalytic

¹ 1 μg = 1 γ = 0.001 mg.

reduction yielded an isomer of estrone (20). The complete synthesis of equilenin from 7-methoxy-1-keto-1,2,3,4-tetraphenanthrene has been reported in a preliminary note (21). An attempt to synthesize the ring system of equilenin has been reported but without success thus far (22, 23). Two new weak estrogenins, 7-ketoestrone and 7-hydroxyestrone, have been prepared from equilin. By oxidation of equilin acetate with osmium tetroxide, hydrolyzing and distilling the resulting 7,8-dihydroxyestrone under diminished pressure, 7-ketoestrone was obtained. The enol diacetate of the latter was converted into the 7-hydroxyestrone (24). Several new synthetic estrogens of very low activity have been synthesized (25).

Metabolism of estrogens.—Earlier attempts to reduce estrone to estradiol by biological reduction always led to negative results. Better results were obtained when esters of estrone are incubated with fermenting yeast (26). The formation of free estradiol indicates that the hydrolysis must proceed in parallel with reduction of the 17-keto group. More recently a 50 per cent conversion of estrone into α -estradiol has been accomplished by incubation for seventy-two hours with sterilized yeast fermentation mixture, adding freshly fermented and sterilized yeast culture every twenty-four hours (27).

That the liver probably is involved in the inactivation of estrogens is indicated by the inactivation of estradiol and estriol by incubation with liver slices, but not by lung, spleen, or uterine tissue (28). The observation that carbon tetrachloride poisoning of immature rats leads to increased uterine weights on about the third day of poisoning has been interpreted as proof that loss of liver function results in less inactivation of blood estrogens and hence greater uterine growth (29). However, whether the loss of inactivation means loss of the power of conjugation with glucuronic acid or loss of the power to destroy estrogens has not been determined.

Physiological effects from estrogens in the female.—Estradiol produces a marked water retention in the tissues of immature female rats. The water content of the uterus, skin, and vagina rises in the first six hours, then falls until the twenty-fourth hour and again rises (29). Treatment of ovariectomized rats with high doses of estradiol dipropionate or estradiol benzoate butyrate decreased the rate of body growth, hastened involution of the thymus, caused hyperplasia of the pituitary, and sometimes resulted in pronounced enlargement of the liver. The effects from estradiol benzoate butyrate persisted for one hundred six days after cessation of injections (30). Hypertrophy of

the pituitary in the rat has also been produced by others through prolonged treatment (31). Large doses of "folliculin" in addition to causing a loss in body weight produce a marked hypertrophy of the thyroid (32).

Estrogens increase the concentration of acetylcholine-like substances in uteri of rabbits if given in doses of 100 to 400 μ g. per kilo body weight. The maximum effect is observed one hour after the injection (33, 34). In gonadectomized rhesus monkeys estrogens cause a marked fall in vaginal pH and progesterone a rise. Uterine bleeding in the normal monkey is usually preceded by a rise in pH (35). Similarly in normal women the vaginal pH values rise to a peak value during menstruation and ovulation (36).

Additional observations on blood calcium changes indicate that large doses of estrogens given for three or more weeks cause a 30 per cent rise in blood calcium within three months in guinea pigs and a prompt rise in cocks and capons (37, 38). One observer associates the rise in blood calcium with changes in the nervous system and an increased resistance to narcotics and toxins (37). In capons or cocks hypercalcification and hyperlipemia were observed in parallel with the hypercalcemia (38). An injection of 300 to 400 units of α -estradiol benzoate caused a hyperglycemia in normal chloralosed dogs, but a hypoglycemia in the adrenalectomized animals. The hyperglycemia due to pancreatectomy was not affected (39).

Actions of estrogens in the male.—Estrone stimulates the development of the rat uterus muscle more than the seminal vesicle musculature in tissue cultures (40). This action *in vivo* is most marked in the young males (41) and it can be partially prevented by progesterone and completely by testosterone (42). Implanted estradiol tablets retard growth in young male rats, cause hypertrophy of the adrenals and pituitary, and produce tumors (43). In the cock prolonged treatment with estradiol benzoate causes a very striking hyperlipemia and hypercalcemia (38, 44). This is considered a "feminizing" effect because the hypercalcemia is a periodic event in the laying hen. Other effects observed in the cock are extreme atrophy of the testes and comb and thyroid pathology (45).

Diethylstilbestrol.—The remarkable similarity in physiological action of diethylstilbestrol and natural estrogens is confirmed in most instances. This parallelism is found in smooth muscle inhibition (46), in effects on the embryo chick (47, 48), inhibition of comb growth (49, 53), retardation of growth of skeletal muscle (50), re-

duction of thymus weight (51, 52), feminizing influence on cock feathering (53), and in sensitizing the infantile uterus to progesterone action (54). Stilbestrol is stated not to cause comb regression when applied on the comb (53). It also does not bring about the rapid rise in acetylcholine-like activity in uterine muscle so characteristic of estrone action (34). Like estradiol benzoate it retards the formation of new erythrocytes in the dog (56, 57). In toxic doses it causes degenerative changes in the liver and adrenals (56, 57, 58).

PROGESTERONE

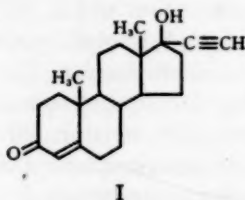
Preparation of progesterone and related compounds.—Progesterone has been obtained in good yield from cholesterol with isodehydroandrosterone as an intermediate. In one process the isodehydroandrosterone acetate is converted into the 17-cyanohydrin derivative, this is then dehydrated in pyridine by phosphorus oxychloride to 17-cyano-3-aceto-5,16-androstadiene. This, after boiling for forty-six hours with methyl magnesium bromide in ether, results in a 75 per cent yield of $\Delta^5,16$ -pregnadiene-3-ol-20-one. Hydrogenation of the latter with Raney nickel catalyst in alcohol produces Δ^5 -pregnene-3-ol-20-one which is easily converted into progesterone by the usual procedures. If the $\Delta^5,16$ -pregnadiene-3-ol-20-one is oxidized in cyclohexone by aluminum isopropylate a new 17-dehydropregesterone is obtained (59). In another procedure the isodehydroandrosterone acetate is treated at 0° C. with the ethyl ester of α,α -dichloropropionic acid in the presence of amalgamated magnesium to produce a chlorohydrin ester androgen derivative which, by careful treatment with sodium hydroxide in methyl alcohol, yields 3(*trans*)aceto-17,20-oxido- Δ^5 -bisnorcholeonic acid ethyl ester. The free acid when heated with quinolin loses carbon dioxide and forms a mixture of pregnenolons which when oxidized in the usual manner yield progesterone and neoprogesterone. The two pregnenolons and progesterone probably involve the *trans*, *cis*-isomerism on carbon seventeen (60). The more direct preparation from cholestenone is still an uncertain procedure (61).

Many new syntheses and derivatives have been made in the pregnene and pregnane series. Some of these will be referred to in the discussion of the cortical hormone. *Epi*- Δ^5 -pregnen-3-ol-20-one, a third form of pregnenolone, has been prepared by the hydrogenation of Δ^5 -pregnene-3,20-dione with Raney nickel catalyst. The mixture of the two Δ^5 -pregnenolons obtained was separated by digitonine pre-

cipitation (62). The preparation of Δ^{16} -*allo*-pregnene-3,20-dione, an isomer of progesterone, and its physiological inactivity is of particular interest because it is another illustration of the specificity of progesterone (63).

The hydrogenation of pregnanediones into pregnanolones and pregnanediols has led to very interesting information on the formation of α - and β -3- and 20-ols. When pregnanedione or *allo*pregnanedione is partly reduced to pregnanol-3-one-20 and *allo*pregnanol-3-20-one, acid favors the formation of the -3- β -ol and absence of acid that of the -3- α -ol form. In the formation of pregnanediols the same is true for the 3-ols but only 20- β -ols are formed (64). The conversion of *allo*pregnanedione into the pregnene series by bromination and debromination by pyridine leads to the formation of a pyridine-2-bromo-*allo*-pregnanedione. The destructive distillation of this complex yielded approximately equal amounts of progesterone and $\Delta^{1,2}$ -*allo*-pregnenedione. The latter is obviously another isomer of progesterone (65). It is also physiologically inactive. Further confirmation of the structural relations between the pregnanediols and bile acids has also been obtained (66).

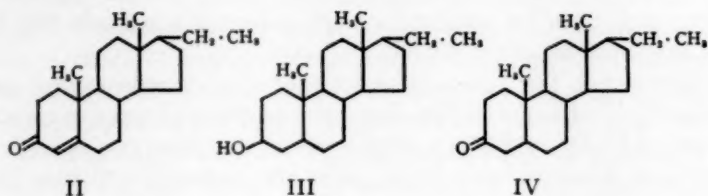
A new progestin has been prepared from the androstene series. Δ^4 -Androstene-3,17-dione is converted into the ethyl enol ether and this into the 17-ethinyl-androstene-3,17-diol-ethyl-ether, by the action of acetylene in liquid ammonia. Warming with acid produced the 17-ethinyl-androstene-3,17-diol or pregnenin-17-ol-3-one (I) (67).



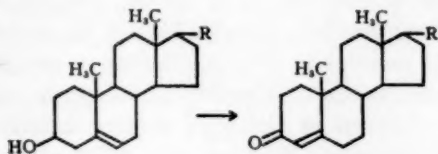
This product is active by mouth in the rabbit in 4 mg. doses. Subcutaneously, 2 mg. are equivalent to 0.6 mg. progesterone in the rabbit (68).

New and confirmatory observations on the structural relations in the sterol, androgen, and pregnane series have been made. From stigmasterol, 20-*allo*pregnanone has been obtained (69), pregnane has been prepared from dehydroandrosterone (70), and Δ^4 -pregnen-3-one

(II), pregnan-3-ol (III), and pregnan-3-one (IV) from pregnan-3-ol-20-one (71).



Of special biological interest is the bacterial oxidation of Δ^5 -pregnen-3-ol-17-one to progesterone because it is another illustration of the general reaction in the androgen series as indicated below (72).



Assay methods.—Advances have been made in improving the sensitivity of progesterone assay methods. In immature female rats the injection of 2 μ g. estradiol benzoate with progesterone in doses of 0.0025 to 0.25 μ g. produces contracted uteri whereas without the progesterone all the uteri are dilated (73). By producing a pseudo-pregnancy in one horn and comparing the other therewith after the injection of progesterone a "two plus" response in endometrial changes is obtained in three days on 0.25 mg. per day (74). By sensitizing the rat uterus with estradiol benzoate, progesterone treatment renders it more susceptible to contraction by adrenalin. An average total dose of 0.41 mg. progesterone causes 50 per cent of the uteri to contract to definite doses of adrenalin (75). Several methods involve the local application of the progesterone to the immature rabbit uterus. The levels of progesterone required to bring about graded responses by such methods range from 0.125 to 0.25 μ g. of progesterone (76, 77).

Physiological action of progesterone and related compounds.—Rabbits spayed on the eleventh day after impregnation require 2 mg. progesterone per day from the eleventh to the fifteenth day and 4 mg.

per day from the sixteenth to the twenty-eighth day inclusive for normal gestation (78). In spayed pregnant rats androgens have been substituted for progesterone with success (79). Among other substitutes suggested for progesterone are desoxycorticosterone (80, 81, 82), pregneninolon-17,3 (68, 83).

In view of the progestin activity of desoxycorticosterone and pregnenin-17-ol-3-one and the structural relations of these to corticosterone and testosterone it is not surprising that these two substances have been found to have slight cortin and androgen activities (84, 85, 86). Progesterone-like cortical extracts have been shown to increase the glycogen content of the liver in rats and fasting ferrets (87).

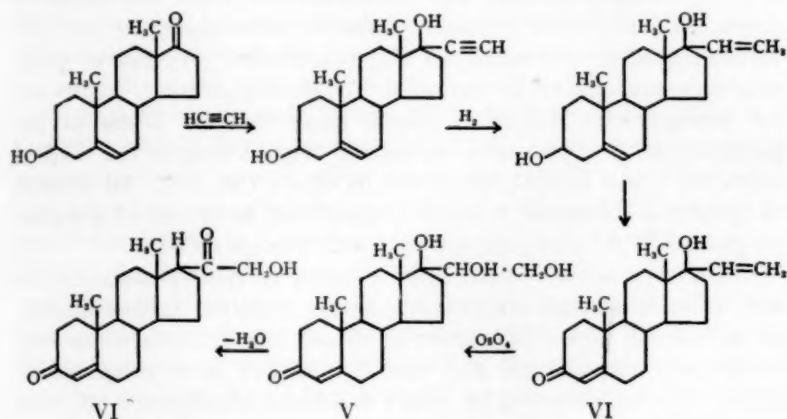
Metabolism of progesterone. Excretion of pregnanediol.—It was a surprise to find that normal men and male patients with Addison's disease, after the administration of 30 mg. progesterone daily, excreted sodium pregnanediol (glucuronide) in the urine (88). Obviously the site of conversion of progesterone to pregnanediol is not limited to the endometrium. Possibly this explains some of the irregular results obtained in the high urinary excretion of sodium pregnanediol glucuronide. This was favorably influenced by intensive estrogen therapy (90). Others report more uniform results (91). According to these investigators nonpregnant women excrete significant amounts of pregnanediol glucuronide during the luteal phase of the menstrual cycle in most cases. It was also found in the urine in the estrogenic phase when late proliferation was found in the endometrium. A continuous daily excretion of 10 mg. or more is indicative of pregnancy and for women who menstruate fairly regularly the excretion of 4 mg. or more of pregnanediol in twenty-four hours after missing a period may be considered fair evidence of pregnancy. In the first two months of pregnancy the excretion rate is 4.7 to 11.7 mg. per day and in the last month it is 27.3 to 73.5 mg. per day. After delivery the excretion rate falls rapidly to a maximum of 6 mg. per day.

SUPRARENAL CORTEX

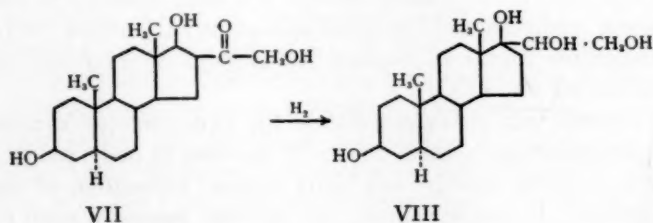
Natural and synthetic hormone preparations.—Six crystalline substances, in part identical with products previously separated by others, and one amorphous fraction have been prepared from cortical tissue. Comparative assays on these showed that some of the crystalline products were inactive while others assayed 0 to 4 rat units per

mg., but the amorphous preparation assayed 20 to 30 rat units per mg. (93).

In the synthetic field new products have been produced which are related to or which add to our knowledge of the detailed structure of various products separated from cortical extracts. A product probably closely related to substance E (Reichstein) has been synthesized in the pregnene series. It is Δ^4 -pregnene-17,20,21-triol-3-one (V). It is physiologically inactive (94). However, when the product is dehydrated it yields desoxycorticosterone (VI) (95). The steps involved in this synthesis beginning with isodehydroandrosterone are:



The same investigators also prepared β -allopregnane-3,17,20,21-tetrol from isoandrosterone; the product proved to be the same as Reichstein's substance K (VIII) which had previously been obtained from substance P (VII) by reduction (96).



Excellent work has been done to clear up the isomerism of the 17-ols of the pregnane and pregnene series derived from adrenal cortex. It

now appears fairly certain that all of these are of the 17(β)ol series. By starting with *trans*androsterone the *allopregnane*-3,17(β)diol and *allopregnane*-3,17-(α)diol were prepared and separated in pure form as acetates and as the diols. The 17(β)diol when oxidized yielded 3-*trans*-17(β)dihydroxyaetiocholic acid, the identical acid produced by oxidizing Reichstein's substance P. Furthermore, by more extensive degradation the 17(β)diol was oxidized back to *trans*-androsterone. In the same way starting with *transdehydroandrosterone* the corresponding 3,17(α) and 3,17(β)diol of the Δ^5 -pregnene series were prepared and from the 3,17(β)diol the 3-*trans*-17(β)dihydroxyaetiocholic acid was produced, but when the oxidative degradation was more extensive *transdehydroandrosterone* was reformed. Furthermore all of the 17(β)ols whether pregnane or pregnene were precipitated by digitonin. The corresponding 17(α)ols are not precipitated. This is conclusive proof that the 17-ols of the pregnane and pregnene series of cortical origin belong to the 17(β)ol series (97), and is partly confirmed by others (98, 99). An attempt to synthesize substance J, which is considered to be one of the *allopregnane*-3,17,20-triols, did not meet with success (100).

Relative activities.—Comparative assays on desoxycorticosterone and cortin in adrenalectomized rats by the muscular fatigue method show that the former is inactive by mouth, but is active when subcutaneously administered and that it possesses some progesterone action. "Cortin" is active by mouth in adrenal insufficiency but does not exhibit progesterone activity (101). In adrenalectomized dogs kept on a low sodium and chloride diet, the daily injection of 1 to 1.5 mg. desoxycorticosterone, dissolved in oil, maintains a 10 kg. animal in good condition (102). Progesterone has been reported to be cortinomimetic in doses of 1 to 4 mg. per day in adrenalectomized rats (103, 104, 105). Closely related pregnane derivatives, various androgens, estrogens, and bile acids showed no such action (104). In fact androgens appear to decrease the average survival time of adrenalectomized rats (105).

In patients with Addison's disease the intramuscular injection of desoxycorticosterone acetate results in increase in body weight, rise in blood pressure, increase in plasma volume, restoration of normal blood plasma, potassium, sodium, and chloride, increased renal excretion of potassium and inorganic phosphate and improved muscular strength (106).

Electrolyte control.—When "whole adrenal extract" is adminis-

tered to adrenalectomized dogs the plasma sodium is maintained at normal levels, but purified cortin alone does not maintain the electrolyte balance unless the "sodium factor" found in cruder extracts is added (107).

Carbohydrate metabolism and cortical extracts.—Starvation of hypophysectomized rats for eight hours lowered the liver glycogen content markedly, but if the animals are injected with desoxycorticosterone for a week prior to the starvation, the loss of liver glycogen is avoided (108). Cortical extract decreases glucose oxidation, increases deposition of liver glycogen (109) and lowers the hyperglycemia produced by adrenalin (110). In adrenalectomized rats higher doses of desoxycorticosterone are required to keep the blood glucose at normal levels than for maintaining normal nonprotein nitrogen and plasma electrolytes (111).

Effects from adrenalectomy.—Adrenalectomy in rats leads to a greater bisulphite binding power in blood (112) and results in lowered sensitivity to the pressor effect from renin (113). Additional evidence has been presented that in adrenal insufficiency the tissues also suffer the changes in electrolytes found in extracellular fluids (114). In fact the chloride concentration decreases more rapidly in the red cells than in the plasma (115). In adrenal insufficiency the renal tubules fail to reabsorb the sodium adequately from the glomerular filtrate even when the serum sodium is low. Administration of sodium chloride improves the reabsorption, but for complete recovery of the renal tubules adrenal extract is necessary (116). Adrenalectomized rats show a craving for salt. It is thought that this is due to changes in the taste mechanism in the oral cavity (117).

ANDROGENS

Preparation of new derivatives and isomers.—Among the isomers the following should be referred to: An isomer of methyl testosterone, Δ^4 -17-hydroxymethyl-androsten-3-one, has been prepared from methyl-3-hydroxy-5-aetiocolenatate by reduction of the carboxyl group to a primary alcohol and subsequent bromination, oxidation, and debromination (113). An isomer of testosterone, Δ^5 -androsten-17-ol-7-one, has been prepared from *trans*dehydroandrosterone by treatment with thionyl chloride to form 3-chloro*trans*dehydroandrosterone, then reduction to Δ^5 -androsten-17-ol and oxidation to Δ^5 -androsten-17-ol-7-one (114).

Attempts to obtain an androgen corresponding to estriol led to the preparation of 16-hydroxy-testosterone. Starting with isodehydroandrosterone by the interaction with methylethylketone in presence of sodium amide and subsequent dehydration a new Δ^5 -androstene-3-ol-

V

17-one derivative with $C_2H_5 - \overset{\text{V}}{\text{C}} - CH_3$ group (at C_{16}) is obtained. This after acetylation, bromination, oxidation by ozone and debromination gave Δ^5 -androstene-16,17-dione-3-ol-acetate. This was reduced to the androstene-3,16,17-triol which condenses with acetone in the presence of hydrochloric acid to an acetone and the latter after appropriate oxidation yields 16-hydroxy-testosterone (115). The substance is water-soluble and possesses slight androgenic and estrogenic activities. New Δ^1 -androstene, *allopregnene* derivatives and Δ^1 -cholestenone have been prepared as a result of the discovery that boiling 2-bromo-derivatives of the *allo*-3-one saturated series in collidine leads to the introduction of the 1,2 unsaturation (116). Various 17-chloro derivatives of estrogens and androgens have been prepared. The 17-chloro- Δ^5 -androstene-3-ol-acetate, 17-chloro- Δ^4 -androstene-3-one and testosterone phosphate prepared in these studies are androgenic when tested by direct application on the comb. The latter is very active (117). The oxidation of cholesterol and *transdehydroandrosterone* by osmic acid leads to the formation of 3,5,6-triols of the *cischolestane* and *cisandrostane* series. From these *cischolestane*-3,6-dione-5-ol, Δ^4 -androstene-3,16,17-trione and androstane-3,16,17-trione have been prepared (118). The fact that conversion of Δ^4 -androstene-3,17-dione to testosterone is accomplished by biological methods (123) led to attempts to carry out the specific reduction of the 17-keto group by other chemical methods. A 70 per cent conversion into testosterone was accomplished by aluminum tertiary butyrate in butyl alcohol and benzene (119).

The action of light on androstenes and pregnenes has produced new compounds. The irradiation of $\Delta^{5,7}$ -androstadiene-3,17-diol by ultraviolet light changes its absorption spectrum in a manner very similar to ergosterol. The resulting compound is, however, not antirachitic (120). If the diacetate of the original compounds is irradiated in the presence of eosin, a diacetate of a peroxide is formed (121). A general reaction of $\alpha:\beta$ unsaturated 3-keto steroids, when exposed to ultraviolet light appears to be the production of difficultly soluble, higher molecular weight compounds with high melting points. The products have not been fully identified (122).

Separation of androgens and related compounds from urine.—

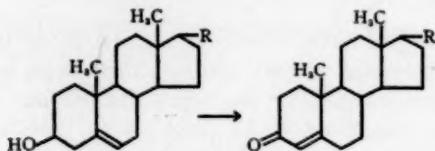
From women's urine, androsterone, dehydroandrosterone, and aetiocholan-3(α)-ol-17-one have been prepared (123). Androsterone and aetiocholan-3(α)-ol-17-one have been separated in larger amounts from human urine after treatment with testosterone propionate (124, 125).

Even in urine from ovariectomized women the androgenic activity is due to androsterone, dehydroandrosterone, and aetiocholanolone (126). These studies indicate that androsterone and dehydroandrosterone may be considered to be products of metabolism of testosterone, but they also indicate that the metabolism is not specifically in the direction of forming physiologically active substances and that the gonads are not necessary for the formation of these substances in the human body.

A new androstane derivative has been separated from the neutral fraction obtained from the urine of pregnant mares. It has been shown to be an androstanolone. It is precipitated by digitonin, gives a very faint Zimmermann reaction and when oxidized yields an androstanedione which is not identical with androstane-3,17-dione. It is tentatively referred to as androstan-3(β)-ol-one (127).

Biological reduction and oxidation of androgens.—The early studies on the action of tissues and microorganisms on various androgens (128, 129) have been extended to include other steroids and the microorganisms involved have been identified to some extent. The transformation of testosterone into aetiocholan-3,17-diol and *epi*-aetiocholane-3,17-diol by extracts from stallion testicles (130) has been considered to be due to microorganisms and not to enzymes in testis tissue (131). The first attempts to reduce androstenedione and testosterone by pure cultures of *B. fluorescens* or *B. coli* in a meat broth medium led to negative results (132). The dehydrogenation of dehydroandrosterone by yeast cultures also proved to be difficult to reproduce (133). A boiled extract of testis tissue alone is inert in the hydrogenation of androstenedione, but very active if inoculated with putrefactive bacteria from successful incubations (134). In the same way sterilized, buffered yeast-sucrose cultures when inoculated with active cultures were very active under aerobic conditions in the conversion of dehydroandrosterone providing the incubation is prolonged and successive additions of saccharose are made (135, 136). Nevertheless, the enzymatic conversion of androstenedione into androsterone by aqueous testicle extracts is still claimed (137). It has

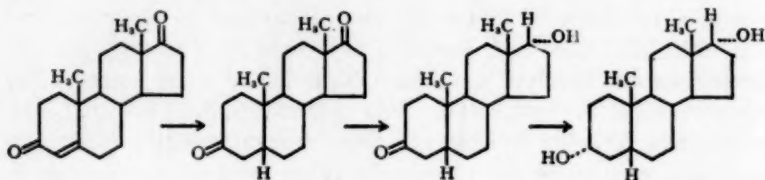
now been well established (138, 139, 140) that a strain of *Corynebacterium* grows very well in a sterilized yeast culture and easily oxidizes under aerobic conditions, the 3-*ol* position in the androstene and pregnene series to 3-*ones*. The general reaction is:



where R may be = 0, $\begin{matrix} \text{CO} \cdot \text{CH}_3 \\ \diagup \quad \diagdown \\ \text{H} \end{matrix}$, $\begin{matrix} \text{CH}_3 \\ \diagup \quad \diagdown \\ \text{OH} \end{matrix}$, or $\begin{matrix} \text{CO} \cdot \text{CH}_2\text{O} \cdot \text{CO} \cdot \text{CH}_3 \\ \diagup \quad \diagdown \\ \text{H} \end{matrix}$

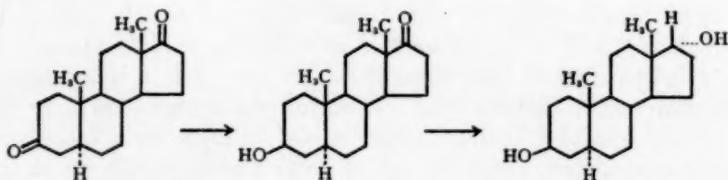
However, cholesterol is not acted upon by the organisms.

The reduction of 3- and 7-keto steroids is likewise due to a strain of *Bacterium putrificus* in buffered yeast cultures under anaerobic conditions. The specificities of the types of hydrogenation are illustrated below for the androstene and androstane series.



Androstenedione-3,17

Epitiocholenediol-3,17



Androstanedione-3,17

Isoandrostanediol-3,17

It should be emphasized that the unsaturated series do not form the *allo* form and the iso-3-hydroxy forms, but the saturated *allo* series

yield the iso-3-ol forms. The 17-keto position in estrone is reduced by microorganisms, but the 3-keto group in cholestenone or cholestanone does not appear to be acted on. The importance of avoiding bacterial action on biological material intended for assays cannot be overemphasized.

Assay methods.—The comb-growth response still is the most generally accepted test for androgenic material. Various modifications of the chick method have been devised (141, 142, 143, 144). The writer feels that much needs to be done to eliminate many sources of error in the baby chick method. The advantage of the chick method by the direct application method is that it is very sensitive, but its accuracy on pure and impure androgens is not well established. The increase in size of the hen's comb has again been suggested as a reliable measure (145). It would appear that the use of any animal with intact gonads must introduce undesirable and obvious complications into the assay method.

Colorimetric method.—The Zimmermann *m*-dinitrobenzene color reaction has been investigated with pure androgens and a number of comparative studies have been made on urine extracts by the colorimetric and biological methods (146, 147, 148, 149). These studies show that all 17-keto androstanes and androstenes give the color reaction, that the two common urinary androgens, androsterone and *trans*-dehydroandrosterone, give the same color value per molecule, and that it is very likely that other 17-keto isomers which may be entirely inactive physiologically all give the same color value. Obviously the colorimetric method is likely to give too high results. Unpublished results in the writer's laboratory confirm this conclusion.

Quantitative extraction of androgens.—Complete hydrolysis of conjugated forms and complete extraction continue to be problems for study. The continual agitation of the benzene-urine mixture in an apparatus using the counterflow principle is recommended (150). Another laboratory recommends two procedures. In one the urine is acidified to a pH 1 after it has been brought to boiling. After one hour's refluxing the extraction is done with benzene in the usual way. In the alternate procedure the properly acidified urine is boiled under reflux condenser with carbon tetrachloride for two hours. The treatment with fresh carbon tetrachloride is repeated twice (151).

Relation of rate of renal excretion of androgens to age, sex, and physiological condition in man.—By the colorimetric method the normal values for adult men are now reported to range from 1 to 3 I.U.

per kg. body weight (152), whereas previously the same laboratory reported a range of 22 to 200 I.U. per liter of urine for adult men and 14 to 65 for adult women (153). These values are too high in terms of biological units because they are based on the colorimetric method and in the calculation the assumption is made that all of the material reacting with the Zimmermann reagent is androsterone. In children the colorimetric method also gave high results as compared with the biological method. Thus in a boy at six years and three months, the daily excretion was 4 to 7.8 units and at twelve years and two months, it was 19 to 31 units. In a girl at seven years and seven months, the range was 4.5 to 8.5 units and at twelve years and five months it was 12 to 18 units per day (154). Other observations based on the same calculations by the colorimetric method indicate an excretion of 40 to 150 I.U. per day in normal women, in patients with benign hirsutism and adrenogenital virilism, 10 to 290 units, and in adrenocortical tumors, 450 to 3,250 units per day (155). The results are of comparative value and certainly indicate an accumulation of 17-keto steroids in the urine in these patients.

In cases of basophilism without adrenal tumors both the colorimetric and biological methods gave low rates of excretion of androgens, but in the cases where adrenal tumors were found, both methods gave high values and *transdehydroandrosterone* was actually separated in larger amounts than from normal men's or women's urine (156). The comparative values by colorimetric and biological assays on urine from patients show very striking fluctuations. By expressing the 17-keto steroids in terms of International Units by assuming 1 mg. 17-keto steroid equivalent to 10 I.U. we observe the following ratios from one publication. In thyrotoxicosis we have the colorimetric over biological units ratios of 104/49, 66/>44, 50/2; in psoriasis 123/53, 72/46, 111/34, 69/7; in haematemesis 130/58, 66/28; in melaena 35/5; in obesity 144/31, 40/25; and in duodenal ulcer 126/20. In other words, the quotients range from 1.5 to 25 (157). On the other hand another group reports the rather low values of 14 to 34 I.U. per day by the colorimetric method in women. The excretion rates were very irregular and frequently higher after hysterectomy and oöphorectomy than before the operation (158). Injection of testosterone propionate in some of the women patients was followed by a distinct increase in androgen excretion (158). All investigators on hypogonadal men and castrates agree that the low rate of excretion of androgens is increased by testosterone propionate

therapy (159, 160, 161, 162, 163). An increased renal excretion of androgens following testosterone propionate injections has also been observed in the immature monkey (164), in the dog (165), and in the rabbit (166). However, the percentage recovery was of very low order as compared with humans, especially in the dog and rabbit. In the studies on man an increased excretion of estrogens follows the male hormone therapy. In acne an increased excretion of androgens is claimed (167). Exposure of man to ultraviolet light and especially if the light is limited to the genital region is reported to increase the androgen excretion by 120 to 200 per cent although the pretreatment level may have been in the upper range of normal values (168). This should be confirmed by biological assays because the colorimetric method was used. A confirmation might help to throw light on the wide fluctuations in the excretion of androgens from day to day.

PHYSIOLOGICAL ACTION OF ANDROGENS

Studies on surviving tissues in vitro.—The male accessory sex organs of the normal and castrated rat or guinea pig react differently as contractile tissues *in vitro* to various drugs. The increased sensitivity to contraction stimuli observed in the castrated rats' accessories is restored to the normal threshold by preliminary treatment of the castrate animal with androgens. Estrogenic treatment exaggerates the castrate type (169, 170, 171, 172, 173) making the *vasa deferentia*, seminal vesicles, and prostates more excitable and reactive.

Effect on metabolism.—Castration of the young rat produces increased renal excretion of nitrogen, sulphur, chloride, sodium, and potassium (174). This confirms the earlier reports as to the opposite effect from the administration of androgens in dogs and man. This has been confirmed on castrated rats (175), but in young rats high doses (1 mg.) for twenty-six to eighty days causes slower growth (176). In castrated rats the increase in body weight produced by testosterone propionate is paralleled by an increased retention of exogenous creatine by skeletal muscle (177). Testosterone propionate also raises the phosphagen, glycogen, and adenylypyrophosphate content in heart muscle of castrated rats to normal values (178). In senility it does not increase muscular activity (179).

Effects from the prolonged administration in the male.—The prolonged administration of androgens to castrated male rats causes increases in the weights of liver, kidneys, heart, and spleen. In the liver it partly counteracts the harmful effects produced by long treat-

ment with estrogens. The androgens also prevent to a considerable degree the tumorlike hyperplasia of the pituitary produced by long treatment with estrogens (175). In normal adult and senile rats there was no improvement in appearance or behavior, but actually a harmful action on the testes (180).

A remarkable inactivation of androgen has been reported and requires explanation. The implantation of pellets of testosterone propionate into the spleens of castrated rats did not regenerate the accessory sex organs, but when these spleens were transplanted these specific effects returned (181).

Effects from the prolonged administration in the female.—In the adult rat the ovaries develop the "wheel cells" observed as a result of hypophysectomy (182), and atrophic changes in the ovary (183) which, with very high doses in young rats, result in lutein cysts and sometimes in ovarian atrophy without luteinization (184). Similar results are observed in mice (185) and guinea pigs (186). In rabbits relatively large doses did not prevent ovulation in response to human pregnancy urine (187) or prolan (188). In *Xenopus Laevis* methyl testosterone induces ovulation, the dose required depending on the season, lowest in the summer and highest in the winter and spring (189).

In female rats high doses of testosterone propionate produce progestational changes in the uterus (190) and neutralize to a considerable degree the hyperplasia of the hypophysis produced by estrogens. Androgens produce the same effects on the liver, kidneys, spleen, and heart as in males. Some stimulation of uterine hypertrophy is observed (191) in the rat (192, 193). In women and in monkeys high doses of testosterone propionate prevent uterine bleeding (194, 195). In all these and many other investigations changes in the vaginal smears and mucosa to the nonestrous type in the treatment of normal animals or in neutralizing the effects of estrogens have been observed.

Action on the pituitary.—Some of the effects of testosterone propionate on the pituitary body have been referred to above. Studies devised more specifically for information on the pituitary show that 10 µg. of testosterone propionate per day injected from the day of birth into castrated rats prevented the development of castration hypersecretion in the pituitaries, but 2 µg. per day did not do so (196). Large doses in normal young rats again showed the harmful effects on the development of testes, and on the appearance of sperm (196).

In mature spayed rats 200 µg. testosterone propionate per day for fifteen days increased the prolactin content of the pituitary body about 40 per cent (197). In immature female rats doses of 2 to 8 mg. testosterone propionate per day for ten to twenty days caused slight decreases in pituitary weights and degranulation in the basophiles but not in the eosinophiles. Other immature female rats treated for longer periods but on lower levels contained ovaries of decreased weight and the pituitaries histologically more nearly resembled the normal male than the female type (198).

Masculinizing action.—The female *Xiphophorus helleri* when injected twice a week with 0.5 mg. testosterone propionate develops male characteristics in two to three weeks. Similar effects were produced by intraperitoneal injections of urine extracts (199). By keeping this species before differentiation of the gonads in an aquarium containing a suspension of the androgen all of the animals become males (200). Masculinization has likewise been produced in canaries (201), female chicks and hens (202, 203). Similar effects in varying degrees have been recorded in the developing *Amblystoma* (204), in genetic female embryo guinea pigs (205), and in the opossum during very early pouch life (207, 208).

The observations above suggest many others on the antagonism between sex hormones and all of these new observations as well as many old ones lead to the conclusion that the mechanisms involved in these studies are exceedingly complex. A very convenient form in which to study such antagonisms is the cock's and capon's comb. The inhibitory effect of estrogens by injection or local application on the rate of growth of the comb has been confirmed (209, 210). The indications are that the site of action by the estrogen is primarily in the comb and that it does not necessarily involve the pituitary-gonad path.

Adrenal origin of androgens.—Additional evidence is accumulating as to an adrenal origin of some of the androgens. The adrenal hypertrophy observed in castrated male rats involves mainly the zona fasciculata and reticularis. This can be avoided by androgen therapy (211). If the adrenals only are removed in immature rats the development of the prostate is not inhibited. The same is true if the testes only are removed. But if gonads and adrenals are removed the prostate development is inhibited. This suggests some androgenic secretion from the adrenal (212). That the X zone in mouse adrenals may be involved is again suggested by the observations that androgen

injections cause the disappearance of this zone in castrated males and normal females (213) but progesterone had no such effect (213), and the same is true for adrenal cortical hormones (214).

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FAT-SOLUBLE VITAMINS

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VITAMIN A

Characterization.—A series of esters have been prepared and examined again by Mead, Underhill & Coward (1). Spectrographic determinations and Carr-Price tests carried out on the two esters anthraquinone-2-carboxylate and 2-naphthoate gave the following values calculated on vitamin-A alcohol after correction for the absorption of the acid components:

	Anthraquinone 2-carboxylate	2-naphthoate
$E_{1\text{ cm. } 1\%}$ 328 m μ	1,790	1,800
Carr-Price value	85,000	96,000

For vitamin A in the pure state, prepared by fractionation of a concentrate in the Hickman molecular still and crystallization at -75° , they found the following data: $E_{1\text{ cm. } 1\%}$ 328 m μ = 1,820 (in ethyl alcohol); and Carr-Price value = 92,000. Other figures have been reported previously by Karrer and Euler.

Biological.—Morton & Creed (2) showed that feeding of carotene (leaf carotene containing both the α - and β -forms) to fresh-water fishes (perch and dace) gave rise to a considerable increase of the store of both vitamins A and A₂.

The distribution of vitamins A and A₂ in the organs of fish and sea birds has been determined (3, 4). Vitamin A₂, which is practically confined to fish and predominates over vitamin A only in fresh-water fish, shows a distribution over the body, which differs appreciably from that of vitamin A. The ratio of vitamin A to vitamin A₂ is higher in the intestine than in other parts of the body. The large vitamin-A deposits in the intestines of many species of fish were shown to be located in the mucosa and particularly in the tunica propria. The distribution of fat in the intestines runs parallel with that of vitamin A. Because of these observations and the particularity of the mechanism of fat absorption in fish, in which fat proceeds through the mucosal epithelium and not by a lymphatic system, the hypothesis was advanced that vitamin-A esters in combination with protein may assist in the dispersion of fat droplets accumulating in the tunica

propria so that they may be removed through the continuous layer of connective tissue (stratum compactum) and proceed into the blood stream. The enormous variation in the vitamin-A content in the intestines of various species of fish led the authors to suggest that the mechanism of assimilation is different when vitamin A is a major intestinal constituent (sturgeon, halibut, cod, and salmon) and when it is a trace constituent (dogfish). Sea birds have much more vitamin A in their intestines than such animals as the rabbit. Vitamin A₂ has been detected in birds subsisting on fish from inland waters.

By means of molecular distillation, Lovern, Mead & Morton (20) found that halibut intestinal oil extracted under conditions excluding hydrolysis contains at least 95 per cent of vitamin A as esters.

The following miscellaneous observations are of interest: "Cyclized vitamin A" occurs in fish-liver oils (31); vitamin A and carotenoids occur in the olfactory area of the steer (5); and a considerable amount of vitamin A is used up during incubation of the hen eggs (33).

A number of papers concerning nightblindness have been published (6, 7, 8, 9, 23, 32). Manville (6) stresses the fact that this disease not only occurs in A-avitaminosis, but also in different diseases of the eye. This is to be taken into consideration, when nightblindness is used as a criterion of vitamin-A deficiency in man. Only such cases as respond to vitamin-A treatment are to be counted. The results of different investigators obtained by measuring the dark adaptation differ considerably; some investigators have concluded that up to two million units of vitamin A are necessary to cure hemeralopia, while others find a few thousand units sufficient. This is apparently due to the great difficulty in making accurate determinations of dark adaption.

Abbott, Ahmann & Overstreet (10) studied the effect of vitamin A on human blood and they concluded that the differential leucocyte count is of diagnostic value in vitamin-A deficiency in man. Vitamin-A deficiency leads to a decrease in total leucocytes and polymorphonuclear counts, an increase in juvenile cells and large lymphocytes, and degeneration of the granulocytes in man as well as in rats. Keratoconus may be produced in the rat by vitamin-A deficiency (11). The storage of vitamin A in livers of rats made carcinomatous by feeding a diet containing 0.1 per cent 2-amino-5-azotoluene during one year does not take place in the tumors but only in the surrounding liver tissue (12). A-avitaminosis in pigs produced pronounced neurologic symptoms: ataxic walk, spasticity, paralysis, often reduced power of vision, and muscular atrophy (13). Mellanby (34) explains

certain nerve degenerations, caused by lack of vitamin A, for instance those leading to deafness, as a consequence of overgrowth of bone, the base of the skull being particularly liable to overgrowth when vitamin A is lacking in the diet. Moore (14) was able to show that the blindness of a nutritional type associated with a constriction of the optic nerve is due to lack of vitamin A. The development of the constriction of the optic nerve is associated with a stenosis of the optic canal. The problem of overdosage with vitamin A was investigated by Noetzel (27) who found that very severe overdoses with Vogan led to necrotic nephrosis.

Determination of vitamin A.—An important contribution to the determination of vitamin A was made by Moll & Reid (15) and by Grab (16). These authors compared the results obtained by the spectrographic method and by the rat-growth test for saponified and nonsaponified concentrates (Vogan), and for liver-oil preparations which are not believed to contain A_2 . In both laboratories a large number of rats were used.

The result of this investigation was that the nonsaponifiable matter of Vogan or liver oil has only half the activity of the untreated Vogan when referred to the same $E_{1\text{ cm. } 1\%}$ 328 m μ or the same Carr-Price value. The conversion factor with which the $E_{1\text{ cm. } 1\%}$ 328 m μ value is to be multiplied in order to give the number of International units was found to be:

	Grab	Moll & Reid
Saponified product	$1,800 \pm 400$	1,800 to 1,900
Untreated product	$3,400 \pm 600$	3,300 to 3,700

If the value of the product before saponification is to be calculated from an $E_{1\text{ cm. } 1\%}$ 328 m μ found after saponification, then the conversion factor for the untreated product is to be used. The results support the conception that in genuine Vogan or liver oils vitamin A is in the form of esters, a circumstance which also accounts for the greater stability of the nonsaponified products. The esters have a greater biologic activity than vitamin-A alcohol (axerophthol). In order to find out whether a given preparation contains vitamin A in the free or in the esterified form, the authors use the method of Ritsert (17): when the oil is shaken with an equal volume of water-free methanol, the methanol phase will only take up 1 to 5 per cent of vitamin-A esters, but 40 to 60 per cent of the free vitamin-A alcohol. Hickman (30) has also found that esters of vitamin A have a different (greater) conversion factor than the free vitamin.

Mead, Underhill & Coward (1) made biologic determinations on the two esters, anthraquinone-2-carboxylate and 2-naphthoate. Calculated on the free vitamin-A alcohol they found one gram to contain 3,181,000 and 3,424,000 International units, respectively. A determination by means of the pure vitamin A itself was, however, not carried out. Under these conditions (vitamin A in ester form) their conversion factor was found to be about 2,000, a result which does not agree with the observations of the authors just mentioned.

The findings of Moll & Reid and of Grab disagree also with those of Hume (18), who found no significant difference between the activity of saponified and genuine halibut-liver oil. Hume (19) also communicated the results of biologic determination of a fresh sample of the *United States Pharmacopeia* reference cod-liver oil carried out in ten different laboratories. She recommends the continuation of the provisional value 1600.

Sources of error in assaying vitamin A.—Cyclized vitamin A, which apparently has no growth-promoting power, possesses a considerable absorption at 328 m μ and gives practically the same antimony trichloride reaction as vitamin A. This should be taken into consideration (31) in the spectrophotometric analysis of vitamin A. The peroxides of fat-like substances oxidize vitamin A (21) and give rise to a product with unselective absorption at 328 m μ . When oxidation has occurred the total absorption at 328 m μ is too high and gives values in excess of the true vitamin-A content.

Ultraviolet radiation of wave lengths exceeding 300 m μ causes a partially reversible decrease in $E_{1\text{cm}}^{1\%}$ 328 m μ for vitamin-A containing concentrates or oils (22).

Methods for determining vitamin A in organs, eggs, serum, and milk also have been described and discussed (24, 25, 26, 28, 29, 33).

VITAMIN D, RICKETS

A review of the chemistry of vitamin D has been given by Brockmann (1) and of the physiology and pathology by Rominger (10).

Chemistry.—Windaus & Güntzel (15) studied the addition compounds of certain products formed by irradiation of 22-dihydroergosterol besides the compound vitamin D₄ obtained previously. With the mercury arc, a lumisterol₄ was found, but no addition product corresponding to D₁. Illumination with the magnesium arc produced a tachysterol₄ and a suprasterol₄. Werder (18) showed

that the toxic dihydrotachysterin has some antirachitic activity, i.e., about 0.5 per cent of that of vitamin D₂.

Mode of action of vitamin D.—Nicolaysen & Jansen (2) investigated the mode of action of vitamin D in the following way: If vitamin D has a direct action on the bone formation, rats without vitamin D will not be able to form normal bones, even if their blood is richly supplied with calcium and phosphorus. If, on the other hand, vitamin D has an indirect effect only (an influence on the absorption of calcium or phosphorus from the intestine), rats without vitamin D must form as good bones as rats supplied with vitamin D, provided that adequate amounts of calcium and phosphorus are brought to the blood stream in the two groups of animals. They, therefore, examined the bones chemically and anatomically in rats raised with and without vitamin D on different diets, and introduced phosphate subcutaneously into rats receiving a low phosphorus diet. The result was that the density of calcification of the bones appeared to be governed solely by the amount of calcium and phosphorus brought to the blood, but the histological examinations showed that vitamin D in certain respects may influence the architecture of the bones. The ratio of calcium to phosphorus in the bones was found to be independent of the ratio of calcium to phosphorus or the presence of vitamin D in the diet.

The phosphorus metabolism, investigated by means of radioactive phosphorus, suggested that vitamin D acts in aiding the conversion of organic phosphorus into inorganic phosphorus, which is then available for bone formation (3). By means of radioactive phosphorus it was found also that the phosphorus metabolism in the bones is more intense in rachitic than in normal chicks (4). Whether vitamin D has any effect on energy production was examined by measuring the carbon dioxide output in awake and narcotized rats. The reduced energy production in rachitic rats was found to be secondary to decreased motility (5).

Van der Rijst & Arons (6) have observed paralysis and hemorrhage in the spinal cord in 75 per cent of their rachitic rats receiving diet 2965 of Steenbock & Black. It is, however, not certain that this symptom was a direct consequence of the lack of vitamin D.

In the vitamin-D deficiency in chicks, it is impossible to find a ratio of calcium to phosphorus in the diet which will prevent rickets in chicks without supplying vitamin D (17). The quantity of calcium and phosphorus deposited depends on the absolute amount ingested,

but is no regular function of the calcium-phosphorus ratio. The number of rat doses required by chicks was found to be eight to sixteen times as high for vitamin D₂ as for vitamin D₃. It has been suggested (7) that massive doses of calciferol act by decreasing the fecal excretion of calcium.

Relation of vitamin D to dental caries.—Taylor & Day (19) examined a group of ten children in India with severe rickets who were living on a diet low in vitamin D, calcium, and phosphorus. None of them had dental caries. A comparison with eight hundred rachitic children led to the conclusion that lack of vitamin D cannot be the cause, or at least not the only cause, of caries.

Rachitogenic substances.—The nature of the rachitogenic properties of phytic acid (inositol hexaphosphoric acid) in cereals was investigated by Harrison & Mellanby (8). Their findings are quite contrary to the view generally accepted, viz., that the phosphorus in the phytic acid is not available to the organism. They found that phytic acid and neutral sodium phytate exert powerful rickets-producing actions when added to a nonrachitogenic or a slightly rachitogenic diet. The rachitogenic action of sodium phytate is antagonized by adding extra calcium to the diet. In agreement with this, commercial phytin (calcium magnesium phytate) is slightly antirachitic.

These observations (8) agree with those of Palmer & Mottram (9) as far as the antirachitogenic action of phytin and the counteracting of the rachitogenic action of cereals are concerned, but these authors suggest that the rachitogenic action of cereals is due to their low calcium and high phosphorus content.

Biological determination.—A prophylactic method has been described for testing vitamin D on young rats which is based on direct measurement of the width of epiphyseal lines (11). The prophylactic Roentgen method used in the Agricultural College of Sweden has also been described by Westerlund (20).

By means of the *United States Pharmacopeia* reference oil fed to chicks, the ash-content method, as adopted by the Association of Official Agricultural Chemists, was examined in a number of laboratories (12, 13). The value of the oil is 95 International units per gm. when tested with chicks. The individual results ranged between about 88 and 133 International units.

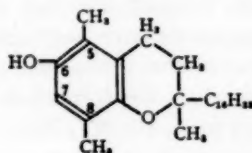
The vitamin-D assay (line test) of low-potency materials such as butter and margarine showed that the large doses of fat, which are required, influence the degree of new calcification indirectly by lower-

ing the intake of salt mixture (14). In such cases care should be taken that the "standard" rats receive the same quantity of the salt mixture as the "test" rats.

Schneider & Steenbock (16) compared the use of a low-phosphorous noncereal diet with the Steenbock & Black diet 2965. They found that vitamin D had a growth-retarding influence when given to the low-phosphorous noncereal diet.

VITAMIN E

Chemistry.—The constitution of natural β -tocopherol was demonstrated by synthesis and by comparison of derivatives with those of the natural compound to be 5,8-dimethyl-tocol (1). From melting point determinations, the so-called γ -tocopherol may be an impure β -tocopherol (27), although it is possible that in γ -tocopherol the methyl



β -Tocopherol

group in the 5-position is lacking (17). Karrer *et al.* (2, 3) synthesized α -tocopherol by means of synthetic phytol and showed that the resulting tocopherol is the same as that obtained from phytol made by saponification of chlorophyll. Alpha-tocopherol is sensitive to light (5). The acetate of *dl*- α -tocopherol is not oxidized by air at room temperature, as is the free alcohol (21).

Relation of chemical features to biological action.—As previously known, β -tocopherol is three to four times less active than α -tocopherol. Karrer *et al.* (1, 3) report that the biological activity in the rat-fertility test does not differ much from β -tocopherol for certain other possible (artificial) dimethyl-tocols—the 5,7 and 7,8 dimethyl derivatives—a result which was also found by Jacob, Steiger, Todd & Work (17). The specificity of vitamin-E activity was further studied by Werder, Moll & Jung (22) and by Evans and co-workers (29).

The activity of different esters of *dl*- α -tocopherol—the acetate, propionate, and butyrate—is higher (about 1 mg. per rat) than that of the free α -tocopherol (2 to 3 mg. per rat) (23).

Chemical methods for the estimation of vitamin E.—The potentiometric titration of vitamin E with auric chloride (5) has been modified so as to correct for carotene and similar pigments. The vitamin E is acetylated before the titration, whereby the hydroxyl group of the tocopherols is protected against oxidation, while the carotene, etc., remains unaltered. The result of the titration after acetylation is to be subtracted from the result of a titration without acetylation in order to obtain the true value for tocopherol. In extracts of animal organs, vitamin A may be destroyed by antimony trichloride. The fact that approximately the same result is obtained with wheat-germ oil as well with as without saponification suggests that vitamin E occurs mainly as the free alcohol.

Boiling of an alcoholic solution of vitamin E with nitric acid yields a red color (4). This color reaction has been recommended for its quantitative determination although catechol derivatives, if present, may interfere. Two suggestions have been made to account for the color: (a) that it is due to the formation of an *o*-quinone (18); (b) that the coloration involves the formation of a *p*-quinone which associates to form a larger molecule (27, 28).

Another colorimetric method has been described, in which vitamin E reduces ferric chloride to ferrous chloride in alcoholic solution in the presence of α,α -dipyridyl (6). This substance forms a red complex compound with ferrous chloride, whereby the ferrous ions are immediately removed from the solution, and the reaction thereby highly accelerated. The color is measured in the Pulfrich Photometer by a weak source of light using screen No. 50. If carotene and similar pigments or vitamin A are present, a correction must be made to eliminate their reducing effect. Adsorption (7) on suitably purified Floridin XS is recommended for this purpose (and for the elimination of vitamin A). This earth is said to adsorb carotenoids and vitamin A, but not vitamin E, from a benzene solution. The reviewer has, however, obtained better results by using the acetylation method of Karrer & Keller (5) combined with the α,α -dipyridyl-ferrous-iron color reaction of Emmerie & Engel (6).

Biological action, symptoms of E-avitaminosis.—Morgulis (26) continued his study of the alimentary muscular dystrophy in rabbits, which has led him to the conception that this disease is a multiple deficiency disease, at least two different vitamins being involved. When either fraction is removed from the diet, muscular dystrophy develops. Cures can be effected by supplying the missing fraction.

When both fractions are incorporated in the basal diet, the disease is prevented. The properties of these two factors make it seem that one is vitamin E, and the other a member of the B-complex, possibly vitamin B₄.

Barrie (8) showed that the neuromuscular symptoms in young rats from mothers receiving a diet low in vitamin E can be prevented by synthetic α -tocopherol. Similar results were independently found by Demole & Pfaltz (9). Dam, Glavind, Bernth & Hagens (10) reported that the encephalomalacia of chicks raised on a diet rich in fat and poor in vitamin E can be prevented by synthetic α -tocopherol. Goettsch & Ritzmann (11) investigated the preventive effect of wheat-germ oils and α -tocopherol in nutritional muscular dystrophy of young rats. They found that α -tocopherol and wheat-germ oil are effective, but oil from wheat germ treated with ferric chloride to destroy vitamin E was also found to be active. They conclude that the activity of a preparation in preventing muscular dystrophy does not necessarily correspond to its antisterility potency. Verzár (25) studied the influence of *dl*- α -tocopherol acetate on the creatinuria in rats with alimentary muscular dystrophy. The creatinuria quickly disappeared after ingestion of large quantities of *dl*- α -tocopherol acetate. A review of nutritional muscle dystrophy up to the appearance of the investigations mentioned here has been given by Morgulis (12).

Action on blood vessels.—Dam & Glavind (13, 14, 15) described a symptom in chicks which they termed "alimentary exudative diathesis." This symptom consists in exudation of large amounts of blood plasma under the skin from the capillaries, mainly of subcutaneous fat tissue. It was shown that this symptom originates from lack of vitamin E in the diet and can be prevented by natural sources of vitamin E and synthetic α -tocopherol. Other known vitamins, including vitamin K and substances which are said to have vitamin-P action, are ineffective. This disease, which is the most striking symptom of E-avitaminosis in young chicks, reveals the importance of vitamin E to the normal permeability of the capillary wall. In contrast to encephalomalacia, the exudative diathesis is not aggravated by incorporating much fat into the diet.

Other effects.—Vitamin E in the form of wheat-germ oil or wheat germ cannot cure fowl paralysis (neurolymphomatosis gallinarum) (16). Deficiency in vitamin E impairs the function of the ovary and influences the thyroid in the same way as castration (19). Demole

(20) could not find any carcinogenic action of vitamin E (synthetic *dl*- α -tocopherol) and concludes that tumors in rats produced, according to some authors, by wheat-germ oil are to be attributed to other factors contained in this oil.

Biological assay.—It has been suggested that the anti-encephalomalacia activity or the antiexudation activity of vitamin E may be used for its biological estimation (10, 14, 15). The rat fertility test has been described in detail by Karrer & Demole (24).

VITAMIN K

Chemistry.—Vitamin K from alfalfa was isolated for the first time by the cooperation of the Chemical Institute of the University, Zürich, and the Biochemical Institute of the University, Copenhagen (1). The substance was a yellow oil of the following percentage composition: C, 82.2; H, 10.7; O, 7.1. It was shown to have a characteristic absorption spectrum with maxima at 243, 248, 261, 270, and 328 m μ . Catalytic hydrogenation destroyed the band at 328 m μ . The extinction coefficient at 248 m μ was 280; it was not altered through repeated chromatographic absorption. Vitamin K yielded an unstable violet-blue color reaction with sodium ethylate, which turned into red and finally into brown. Karrer (2) has given an explanation for the formation of the blue component. Pure vitamin K from alfalfa was found to contain about twenty million of the units per gram (38), *i.e.*, the units used in Dam's laboratory.

Previous claims for the isolation of vitamin K from alfalfa in the form of colourless crystals (3,4) could not be substantiated, and it was shown (1) that the substance melting at 69°, which had previously been claimed by Doisy and co-workers (4) to be pure vitamin K, was an inactive hydrocarbon.

Almquist & Klose (5) reported that they had isolated vitamin K as a choleic acid melting at 186–189° from which the vitamin could be liberated in the form of a viscid oil. They did not, however, put forward any criterion for the purity of the product.

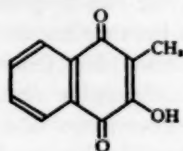
Doisy and his collaborators (6) confirmed the previous finding of Almquist (7) that vitamin K in concentrated form is destroyed by light. In the same paper they mentioned two substances, a crystalline product obtained from fish meal (m.p. 50.5–52.0°) and a non-crystalline product from alfalfa, both of which had high vitamin-K activity (the noncrystalline being the more potent). The activity of both products was destroyed to a considerable extent by exposure to

sunlight or light from a 300-watt bulb. These same authors (6a) later reported that they had isolated pure vitamin K from alfalfa and fish meal, since the substances described in their foregoing paper had turned out to be pure. They proposed to term the noncrystalline vitamin K from alfalfa, K_1 , and the crystalline vitamin from putrefied fish meal, K_2 , a suggestion which has been followed by other authors. Their data for K_1 —absorption bands and percentage composition—were in close agreement with those reported by Dam *et al.* (1). For the absorption at 248 m μ they reported a higher extinction coefficient, viz., $E_{1\text{ cm.}}^{1\%} = 385$. The mol. wt. (Rast) was found to be 443, 464.

From the difference in extinction coefficient between their preparation of vitamin K_1 and the one prepared in Zürich, they claimed that they were the first to isolate vitamin K_1 . In a later paper (17) Doisy *et al.* declared that their first determination of the extinction coefficient was inaccurate; the value should be 540 instead of 385. Details of the ultraviolet-absorption determination reported by the St. Louis workers were given by Ewing, Vandenbelt & Kamm (89). For a further elucidation of this question the reader is referred to the paper of Karrer *et al.* (18) and to Fieser (27), who could not find the high value for the extinction coefficient reported by the St. Louis workers.

The elementary composition of vitamin K_2 was found to be: C, 84.43 to 84.57; H, 9.87 to 9.73; and mol. wt. 552, 561. The absorption spectrum of vitamin K_2 resembled that of K_1 : bands at 249, 261, 269, and 320. Upon hydrogenation vitamin K_1 took up eight atoms and vitamin K_2 eighteen atoms of hydrogen. The colorless hydrogenation products of both vitamins were oxidized to yellow compounds on exposure to air, and these oxidation products could absorb one mol of hydrogen to form the colorless compounds again (6a). So it was concluded that the two vitamins have a quinoid structure. Thayer, McKee, Binkley, MacCorquodale & Doisy (34) report that vitamin K_1 has 1,000 and vitamin K_2 660 of their units per mg. Both groups of investigators agree that vitamin K_1 crystallizes by intense cooling and melts below zero. The quinoid structure was also found by Karrer and co-workers (8). In agreement with Dam, they proposed the name phyllochinon for vitamin K_1 . Detailed descriptions of methods for preparing pure vitamin K_1 from plant material have been given (18, 23). The details of the isolation of vitamin K_2 have also been described (87). The recognition of the quinoid structure re-

sulted in a number of papers (9 to 16, 20) dealing with the anti-hemorrhagic activity of different quinones. The first paper of this kind was that of Almquist & Klose (9) reporting that synthetic phthiocol, 2-methyl-3-hydroxy-1,4-naphthoquinone (the natural product comes from human tubercle bacilli) possessed antihemorrhagic properties. Phthiocol is soluble in water. [Other water-soluble compounds with vitamin-K activity were described later (88), viz., 2-methyl-1,4-naphthohydroquinone and 4-amino-2-methyl-1-naphthol.] Ansbacher & Fernholz (10) found that 2-methyl-1,4-naphthoquinone is practically as active as natural vitamin K₁, that the diacetate of 2-methyl-1,4-naphthohydroquinone is somewhat less active, and that phthiocol is several hundred times less active. Of the quinones tested



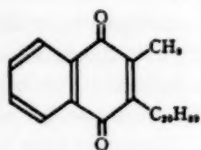
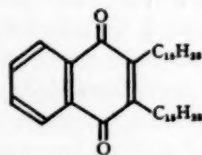
Phthiocol

by Fieser *et al.* (12), 2,3-dimethyl-1,4-naphthoquinone was found to be most effective. Kuhn *et al.* (16) found that α -tocopherol-quinone has some vitamin-K activity, so that the K activity is not strictly confined to naphthoquinone derivatives. Later Ansbacher & Fernholz (90) showed that phlorone (2,5-dimethyl-benzoquinone) has a low K activity. Determinations (91) by the method of Dam & Glavind (38) using 2-methyl-1,4-naphthoquinone as standard substance indicated that the diacetate of the corresponding hydroquinone is the more stable substance. Several papers dealing with the K activity of quinones contained rather conflicting results, the activity indicated for a given compound often being in direct contradiction to activities given by other authors or by the same author in a previous paper. The results mentioned above are some of those which should be considered reliable.

Certain simple derivatives of the natural vitamin K, viz., the diacetates of the corresponding hydroquinones, have been prepared and described [K₁ and K₂ (17); K₁ (8)]. The molecular weight of vitamin K₁ was determined by potentiometric titration with sodium dithionite (Na₂S₂O₄), and at the same time the redox potential was found to be, $E_m = +0.005$ volts.

Doisy and his co-workers (21) were the first to obtain degradation products of the molecule. Ozonolysis of the diacetate of the hydroquinone corresponding to vitamin K_1 yielded a ketone, which probably was 2,6,10-trimethyl-pentadecanone-1,4. This suggested the presence of a phytyl radicle. Oxidation of vitamin K_1 with chromic acid yielded among other products two acids: phthalic acid, and supposedly 2-ethyl-1,4-naphthoquinone-3 acetic acid. These observations would fix the structure of vitamin K_1 as 2-ethyl-3-phytyl-1,4-naphthoquinone. In a later paper (22) however, it was reported that condensation of phitylbromide with the monosodium salt of 2-methyl-1,4-naphthoquinone yielded 2-methyl-3-phytyl-1,4-naphthoquinone, which was identical with vitamin K_1 . These investigations have been further described by Doisy and his associates (86).

Fieser and co-workers (12, 24, 25) approached the problem from another point of view. Having already worked on syntheses with naphthoquinones, as soon as the possible relation of vitamin K to this group of substances was suggested they tested a series of quinone derivatives for vitamin-K activity and found 2,3-dimethyl-1,4-naphthoquinone to be very effective and more potent than the 2,6 and 2,7 dimethyl isomerides. A high potency was also found for 1,4-naphthoquinone having an unsaturated side chain (allyl). Further they compared vitamins K_1 and K_2 with different synthetic naphthoquinone

Vitamin K_1 Vitamin K_2 (tentative)

derivatives with respect to ultraviolet absorption and the reaction with sodium ethylate. The allyl derivatives were found to give a positive sodium ethylate reaction. Their results led to the suggestion that vitamin K_1 should be 2-methyl-3-phytyl-1,4-naphthoquinone, and vitamin K_2 2,3-difarnesyl-1,4-naphthoquinone. After a series of trial syntheses (26) the synthesis of vitamin K_1 was carried out (27) by heating phytol and a large excess of 2-methyl-1,4-naphthohydroquinone in the presence of anhydrous oxalic acid. (The large excess of the hydroquinone and the relatively low temperature served to depress cyclization to naphtho-tocopherol.)

The phytyl-substituted hydroquinone is extracted from ether by dilute alkaline hydrosulphite and oxidized by silver oxide to the quinone. The corresponding ethyl compound was found to be inactive. The identity of the synthesized compound with natural vitamin K₁ was demonstrated by comparison of the diacetates of the hydroquinones (28). Ansbacher, Fernholz & MacPhillamy (95) state that concentrates of alfalfa may be more potent than synthetic K₁, but this has not been affirmed by other authors.

Fieser, Campbell & Fry (25) give a theoretical explanation of the purple-blue color reaction of the natural vitamin K and other β -unsaturated alkyl naphthoquinones with sodium ethylate according to which the unsaturated side chain is replaced by a hydroxyl group. This would lead to the formation of a phthiocol-like pigment. They further suggest that the phthiocol isolated from human tubercle bacilli may have arisen from the alkaline decomposition of a K-type vitamin.

Chemical determination.—Almquist & Klose (29) tested the use of the color reaction with sodium methylate for determining the activity of vitamin-K concentrates and extracts of certain bacteria. They found good agreement with the biological assay. The determination was carried out by dissolving a few milligrams of the concentrate in 1 or 2 cc. of methanol, adding 1 cc. of a methanol solution of sodium methylate (2 to 3 g. of sodium plus 50 cc. of methanol) and warming for a few minutes. When the reaction had reached the red-brown stage, carotenoids were extracted by a hydrocarbon solvent, leaving the characteristic color in the methanol phase. Fernholz *et al.* (30) reported that the color reaction does not run parallel with the biological activity and that the color-yielding substance may be separated from the vitamin by chromatographic adsorption on calcium sulphate. They suggest that the color is due to an easily formed decomposition product of vitamin K. This view is not in accordance with the later observations (2, 25) and probably is incorrect.

It seems likely that a spectrographic method for determination of vitamin K could be developed, but as far as the reviewer is aware no publication on this subject has been issued. Almquist (20) reports that vitamin K₁, containing the phytyl side chain, exhibits fluorescence in the light of an argon lamp, which is not the case with the simple naphthoquinones without the side chain.

Biological assay units.—The methods of biological assay used are mainly those described by Almquist (31), Ansbacher (35, 36), Thayer

(33, 34), Dann (96), and Dam (38) and their co-workers. The four first mentioned have been published within the period of review.

The vitamin-K-free diet proposed by Almquist & Klose (31) is used by several workers. It consists of: sardine meal, ether extracted, 17.5; dried brewer's yeast, ether extracted, 7.5; salt (contains 0.5 per cent manganese as manganous sulphate), 1.0; cod-liver oil, 1.0; calcium carbonate, 0.5; and ground polished rice, 72.5 per cent. The diet is no doubt free of vitamin K, but is deficient in certain other factors necessary for the chick, for instance vitamin E. Almquist & Klose give this diet to day-old chicks for one week. Thereafter, they give diets consisting of the basal ration plus the concentrate to be tested for two weeks. At the end of the test period, they determine the mean clotting time and the mean prothrombin time (32) for ten chicks by means of blood obtained by decapitation. The reciprocal values of these figures are said to bear a linear relation to the logarithm of the vitamin-K level in the diet.

The results obtained with the substance being tested are compared with those of a standard and are expressed in terms of this standard. The standard employed is a hexane extract from alfalfa, of which 1 cc. corresponds to 1 gm. of dried alfalfa and, therefore, probably is equal to 300 Dam units.

Thayer *et al.* (33) give groups of ten-day-old chicks the vitamin-K-free ration of Almquist for fourteen days. The concentrate to be tested, dissolved in 0.6 cc. of sesame oil, is given orally, 0.2 cc. of the sesame-oil solution being administered on each of three successive days, by means of syringe and tube. On the fourth day blood is drawn from the brachial vein for determination of the spontaneous clotting time at room temperature. A clotting time of ten minutes or less is arbitrarily considered normal. The percentage of chicks in the group showing a clotting time less than ten minutes is plotted against the dose. The unit of these authors is defined as the quantity of the vitamin, which produces a clotting time of ten minutes or less in 50 per cent of ten or more chicks. One gram of dried alfalfa is found to contain approximatively ten of the authors' units. One Thayer unit should therefore equal thirty Dam units, but from the figures for K_2 the conversion factor is 13.5. In order to eliminate differences in the degree of deficiency produced in fifteen days in different shipments of chicks the authors determined the response of each batch of chicks to a standard preparation of vitamin K, and they advise that before starting assays with a particular lot of chicks, the clotting time

of ten or more should be determined to ascertain the degree of deficiency. In a later paper (34) the same authors describe the use of a one-dose assay with determination of the clotting time eighteen hours after the ingestion. They found that this method gave satisfactory results.

This group of investigators (14) has proposed to use 2-methyl-1,4-naphthoquinone as reference standard for vitamin K. This substance is, however, sensitive to light and has certain clinical disadvantages. It therefore appears that it would be more suitable to choose a naphthoquinone derivative, which has no such properties such as 2-methyl-1,4-naphthoquinone diacetate (89).

Ansbacher's method is based on the fact that the clotting time of vitamin-K-avitaminotic chicks is gradually reduced during a certain time after the ingestion of vitamin K and thereafter gradually increases. After six hours an approximately normal clotting time may be reached with a sufficiently large dose of the vitamin if introduced directly into the blood stream (37). Ansbacher reported independently that this is also the case when the vitamin is given orally. [Reports indicating normal clotting power as early as one hour after injection of the vitamin (85) are uncertain.] Six hours after the vitamin has been given by syringe and tube into the crop (dissolved in 0.1 cc. of cod-liver oil) the clotting time at 38° to 40° is determined in blood obtained by vein puncture. (Ansbacher stresses the fact that the amount of oil used for the solution of the concentrate should be small.) Under these circumstances the coagulation time of normal chicks is less than six minutes. Five animals are used for each dose of the vitamin. The unit of Ansbacher is defined as the minimum amount necessary to render the blood-clotting time for the vitamin-K deficient chick, weighing 70 to 100 gm. normal (i.e., less than six minutes) within six hours after administration.

Ansbacher has compared his unit and the unit used by Dam & Glavind (38) by testing a sample of their standard by means of the Ansbacher procedure. The Ansbacher unit was found to equal twenty Dam units. Ansbacher's method is a rapid method for determining the activity of concentrates or potent substances, but when dried vegetables and animal organs are to be tested, the rate of absorption may lower the results. This is perhaps the explanation for the fact that a comparison of the activity found by Ansbacher and by Dam & Glavind for 2-methyl-1,4-naphthoquinone indicated that one Ansbacher unit corresponds to 12.5 Dam units. The method of Dann (96)

resembles that of Thayer *et al.* Disadvantages common to the methods of Almquist, Dann, Ansbacher, and Thayer are that the blood is exposed to inconstant admixture with tissue extract when drawn from the vein and that it is dangerous to determine the clotting time before the test substance is given, because of the risk of bleeding.

The ideal method should no doubt fulfill the following demands: The several chicks which are to be tested should have been raised on a diet which is completely deficient in vitamin K, but which contains all other factors necessary for normal development. The determination of the clotting power of the blood or plasma should be made both before and after the administration of the sample. The blood should be obtained without admixture of tissue extract, thereby obviating the induction of coagulation with an indefinite amount of tissue extract. Activities should be expressed in terms of activity of a standard substance, which is easily reproducible and stable to light and oxidation by air.

An idea of the deviation of results reported by different authors for one and the same substance is given in Table I:

New reports on vitamin-K avitaminosis in mammals.—Vitamin-K avitaminosis has been demonstrated in mammals. Murphy (39) has reported that in a group of mice receiving a vitamin-K-free diet, the average bleeding time determined by Duke's method was somewhat prolonged. A light-grade vitamin-K avitaminosis in rabbits has been found (40), and also a severe vitamin-K avitaminosis has been observed in rats, which could be cured by intravenous injection of vitamin K (41). Very marked symptoms were observed in seven out of fifteen rats.

Mode of action.—According to several investigators (32, 76), the function of vitamin K in preventing hemorrhage consists in the maintenance of the prothrombin level in the blood. The way in which vitamin K influences the formation of prothrombin has been studied by Dam, Glavind, Lewis & Tage-Hansen (37) who found that the development of the action requires a certain time (several hours), and that blood from vitamin-K-avitaminotic animals, left in contact with a vitamin-K emulsion at body temperature for several hours did not improve the clotting power. From this they concluded that the interaction of tissue is necessary. The fact that prothrombin precipitations from the plasma of normal chicks did not show vitamin-K activity in the chicken test was interpreted to mean that vitamin K is not a prosthetic group in the prothrombin molecule, but that its presence in the

TABLE I

VITAMIN-K ACTIVITY OF VITAMIN K₁ AND OF 2-METHYL-1,4-NAPHTHOQUINONE

Investigator	Vitamin K ₁		2-Methyl-1,4-Naphthoquinone		
	Investigator Units per mg.	Dam Units per mg.	Investigator Units per mg.	Dam Units per mg.	Potency Relative to K ₁
Almquist & Klose (11)	0.435	130*	
Almquist & Klose (20)	65	20,000	5.15	1,550*	8 per cent of K ₁
Almquist & Klose (46)	63	20,000	240	72,000	Four times as active
Ansbacher & Fernholz (10)	2,000	40,000†	Practically as active
Ansbacher & Fernholz (45)	500	10,000	2,000	40,000†	Four times as active
Ansbacher <i>et al.</i> (95) ..	133	2,660
Dam & Glavind (made for comparison)	12,000	...	25,000	More potent
Dam, <i>et al.</i> (1)	20,000‡
Dann (96)	1,000	2,500	...	More potent
Fieser <i>et al.</i> (26)	6,000	...	25,000§	Four times as active
Thayer <i>et al.</i> (13)	1,000	14,000	10	140¶	1 per cent of K ₁
Thayer <i>et al.</i> (14)	1,000	14,000¶	Same activity
Tishler & Sampson (15)	Same order of magnitude
Sjögren (91)	30,000 to 35,000

* The conversion into Dam units is based on the assumption that the reference standard of Almquist corresponds to 300 Dam units per gm.

† The factor of Ansbacher being used for the calculation of Dam units. Compare, however, the corresponding part of the text.

‡ Later determinations have given 12,000 units per mg.

§ The conversion into Dam units is based on the assumption 1 gm. of Walker standard alfalfa has 300 Dam units per gm.

¶ The calculation of Dam units is based on the assumption that 660 Thayer units [the figure for 1 mg. K₂ (34)] equal 9,000 Dam units (8).

|| Synthetic 2-methyl-3-phytyl-1,4-naphthoquinone, compare text.

tissue promotes the production of prothrombin. It was easy to show by extirpation experiments that the spleen is not essential to prothrombin formation, and that ligation of the pylorus was of no importance, thus indicating that a mechanism similar to that of the extrinsic antiperniciosa factor can be excluded.

The highly important question concerning the role of the liver was very difficult to elucidate because in extirpation experiments with

birds (ganders) the clotting power of the blood plasma improved when tested with tissue extract. This point therefore remained unsettled. Warner (73), however, reported that a partial hepatectomy in rats (removal of about two-thirds of the liver) causes an immediate fall in prothrombin to 25 per cent of the normal level. The action of vitamin K was not tested. Papers concerning the role of the liver are further discussed in connection with the clinical work on vitamin K; and see also (100, 101, 102).

Role of vitamin K in human pathology and its clinical use.—As known to the readers, the year 1938 brought forward the demonstration of vitamin-K avitaminosis in man in connection with obstructive jaundice. This was shown independently by Warner, Brinkhous & Smith (42), Iowa; Butt, Snell & Osterberg (43), Mayo Clinic; and Dam & Glavind (44), Copenhagen. The reviewer takes the liberty of correcting an error in Volume VIII of this *Review* according to which Dam & Glavind did not find any effect of their treatment of patients with intramuscular injection of vitamin K.

The year of 1939 brought a number of papers dealing with the use of vitamin K as a preoperative and postoperative measure to prevent the risk of bleeding in patients with obstructive jaundice (47 to 55 and 57 to 60) and other papers demonstrating the role of vitamin K in other fields of human pathology (61 to 64, 66, 67). Some of those working with obstructive jaundice did not have vitamin-K preparations of sufficient strength to obtain good results. This applies to the work of Illingworth (47) and Rhoads (50). The latter used up to 25 gm. "cerofyl" (a chlorophyll-containing powder prepared from oat plants) which according to the reviewer's determination correspond to about 20,000 Dam units. Apparently this is below the limit necessary for peroral treatment.

The fact that the clinical workers express the activity of their vitamin-K preparations in different ways makes it difficult to compare or evaluate their results. Where the doses have been expressed by comparison with the activity of a certain quantity of dried alfalfa, the dose may, however, be approximately calculated (1 gm. of dried alfalfa contains about 10 Thayer units, 15 Ansbacher units, or 300 Dam units).

There is no doubt that the upper limit of doses indicated by Butt, Snell & Osterberg (56), viz., 240,000 Dam units per day together with 1 to 2 gm. of bile salts, for instance sodium desoxycholate, is entirely adequate—i.e., it may restore the prothrombin level to approximately

normal values in the course of one to two days. It is likely that 1 Dam unit per gm. body weight is the proper dose per diem.

Tage-Hansen (58) reported that a single peroral dose of 100,000 to 200,000 Dam units and 2 gm. desoxycholic acid gave normal coagulation within two days. Some days later the coagulation defect began to set in again. Intramuscular injection of an oil solution of the vitamin in one day or in a three to five day period led to restoration of normal blood coagulation after one to two weeks, using a total of 150,000 to 400,000 units for each patient. In some nonsurgical cases, it could be shown that the effect of large doses of oil solutions given intramuscularly lasted for at least three weeks. Intravenous injection of emulsion (0.5 or 1 cc. containing from 15,000 to 30,000 units in one dose) yielded normal blood coagulation within eighteen hours, but as in the case of peroral introduction the effect only persisted for a few days. All clinicians agree that peroral or intravenous treatment with vitamin K, in order to check the bleeding risk in operations on patients with obstructive jaundice, should be given before as well as after the operation.

Some authors have attempted to use phthiocol instead of the natural vitamin K. Smith *et al.* (52) injected a large volume of solution intravenously, viz., 45 cc. of 0.2 per cent solution of phthiocol in a phosphate solution of pH 7.4, and Butt, Snell & Osterberg (55) injected intravenously 250 cc. of a solution containing 50 mg. of phthiocol. Both groups reported that this treatment was successful and that no untoward reactions were noted. Also 2-methyl-1,4-naphthoquinone (5 to 10 mg.), corresponding to 125,000 Dam units (99), and the water-soluble 1,4-dihydroxy-2-methyl-3-naphthaldehyde (94) have been used successfully.

Koller (60) tested clinically the effect of 2-methyl-1,4-naphthoquinone and 2-methyl-1,4-naphthohydroquinone diacetate, after having investigated the toxicity of very large quantities of these in dogs and rabbits. Administered to patients, both compounds were active in cases of hypoprothrombinemia but large quantities of the quinone, 180 mg. given perorally, led to vomiting and porphyrinuria. A dose of 30 mg. per kg. body weight injected intramuscularly into a dog caused vomiting, albuminuria, and porphyrinuria. The diacetate of the hydroquinone lacked the burning taste of the quinone and did not lead to vomiting or porphyrinuria, but 60 mg. per kg. injected intramuscularly into a dog led to transient albuminuria. He suggested that the acetyl group is not split off before the substance reaches the

intestine and, therefore, the large doses of this compound are better tolerated. The doses used by Koller are extremely high. According to the experiences of the reviewer 5 to 10 mg. of the quinone or 10 mg. of the diacetate given perorally are entirely sufficient for an adult person.

The question whether the deficiency in prothrombin, encountered in patients with obstructive jaundice, is due solely to diminished absorption of vitamin K from the intestine because of exclusion of bile from the intestinal tract, has been answered somewhat differently during the year. At the beginning Snell (65) put forward the view that the ability of the liver to utilize vitamin K in the formation of prothrombin may perhaps be a more important factor. The workers at the Mayo Clinic were apparently at that time of the opinion that such conditions are treatable by great doses of vitamin K. There is some difficulty in accepting this view since practically all cases of hypoprothrombinemia caused by obstructive jaundice respond to treatment with moderate doses of vitamin K. Later (57) it was reported that even a badly damaged liver is actually capable of synthesizing prothrombin. However, certain patients having severe hepatic damage were encountered, who did not present the usual prompt response in prothrombin formation following the administration of vitamin K. In one case the patient showed no response whatever. These observations appear to be in accord with those of certain other workers (51) who reported that reduced prothrombin may be found in cases of certain liver diseases (Lännec's cirrhosis and syphilitic "Banti's disease") and that this form of hypoprothrombinemia does not respond to treatment with vitamin K [cf. also (59)]. In experimental occlusion of the bile duct in rats the hypoprothrombinemia sets in so rapidly that no time is left for the development of liver damage (83, 84).

Most clinical workers take it for granted that the liver is the site of formation of prothrombin and therefore the organ through which vitamin K performs its action. This view is supported by the clinical observations described above as well as by experiments with chloroform intoxication (cf. 72), hepatectomy (cf. 73, 100, 101), and intoxication with spoiled sweet clover (cf. 74).

It is obvious that the reliability of reports of unsuccessful treatment with vitamin K depends on whether the vitamin has been given in quantities sufficient for successful treatment of cases with simple obstructive jaundice.

It appears that these questions and their clinical consequences may not be considered to be completely settled, before the following problems have been fully elucidated.

(a) The possibility of reduced production of bile acid as cause of insufficient absorption of vitamin K in accordance with previous experiments of Smyth & Whipple (75).

(b) The possibility of simultaneous impairment of tissues other than the liver.

(c) The realization of a liver-extirpation experiment under conditions which allow the study of the action of intravenously injected vitamin K or perfusion experiments.

Variation in diagnostic methods.—The methods used by the different groups of investigators for determining the degree of vitamin-K avitaminosis in patients vary very widely. The workers of the Mayo Clinic, Butt, Snell & Osterberg, and many other investigators use the method of Quick (32), in which the time for coagulation produced by a large excess of tissue extract is measured. The results can be converted into prothrombin values by means of a standard curve, but the Mayo Clinic workers prefer to determine prothrombin directly by the method of the Iowa workers, Smith and colleagues. Up to 1939 these used a two-stage method (79), in which plasma from the patient, defibrinated in a particular way, is allowed to act on a fibrinogen solution. From the coagulation time, the prothrombin content may be found by means of a standard curve. During the year of 1939 the Iowa workers (54) devised a "bed-side test" for controlling the action of vitamin K. This method is particularly simple. It is carried out in the following way: In a 3 cc. test tube is placed 0.1 cc. thromboplastin (tissue extract). Blood, freshly drawn, is run into the tube to a 1 cc. mark, then inverted at once, and tilted gently every few seconds, and the clotting time observed. The test is repeated on a normal individual and the unknown expressed in percentage of the normal.

$$\text{Clotting activity (in per cent of normal)} = \frac{\text{clotting time of the normal}}{\text{clotting time of the unknown}} \times 100$$

The results of this new method are said to run parallel with those of the old two-stage method. [But see Quick (102).]

The Copenhagen workers use the method described by Dam & Glavind (40), in which heparin plasma from the patient is brought to clot by varying concentrations of tissue extract, whereby the con-

centration of tissue extract, which yields coagulation in three minutes, is noted. This concentration divided into the corresponding concentration which coagulates a normal plasma under the same conditions, gives the clotting activity, which, so far as other factors are constant, is identical with the prothrombin activity.

$$\text{Clotting activity (C)} = \frac{\text{concentration for normal}}{\text{concentration for patient}}.$$

The reciprocal figure *R* of the clotting activity indicates the coagulation anomaly.

There is reason to believe that the values for prothrombin found by different methods do not agree very well. Thus the Iowa workers indicate that the danger zone (the limit for occurrence of bleeding) is reached when the prothrombin level has fallen below 50 per cent of normal. The Copenhagen workers have never observed a bleeding tendency with a reduction of prothrombin to 50 or 30 per cent of normal. According to the work of Quick (32) a reduction of prothrombin to 30 per cent scarcely influences the clotting time, when an excess of tissue extract is used, and, therefore, can not be recorded by his method in its simple form.

Hult (67) showed that the hemorrhagic diathesis met with in certain cases of sprue is a vitamin-K avitaminosis and can be cured by vitamin K. It had previously been suspected that this symptom might be due to the unfavorable conditions for absorption of vitamins from the intestines (69, 70) and particularly of vitamin K (44, 56, 66, 70).

Clark, Dixon, Butt & Snell (78) studied a series of intestinal disorders, including sprue. They found hypoprothrombinemia, which could be cured by vitamin K, in varying degrees not only in sprue, but also in cases of intestinal polyposis, chronic ulcerative colitis, intestinal fistula, postoperative gastric retention, gastrocolic fistula, and intestinal obstruction. Cases of extreme hypoprothrombinemia without manifest obstruction of the bile duct were found by Dam, Tage-Hansen & Plum (63, 92) in infants with icterus gravis. This hypoprothrombinemia was eliminated by treatment with vitamin K.

Coagulation of infants' blood.—The coagulation power of the blood of newborn infants was studied several years ago. Thus Rodda (80) in 1920 reported that during the first days of life the blood clots more slowly than blood from older children. Brinkhous, Smith & Warner (81) in 1937 reported that this is due to a relatively low prothrombin content. Whipple (82) had already found that the blood of infants

with hemorrhagic diathesis had a very low clotting power. Brinkhous, Smith & Warner found in accordance with this that in such cases the prothrombin was much reduced. During 1939 this problem was studied independently by several authors with a view to a possible vitamin-K avitaminosis, and it was shown that the hypoprothrombinemia of the newborn child could be eliminated by vitamin K.

Waddell *et al.* first reported this result (61, 62). They urge the use of vitamin K before all operations on infants in the first week of life and venture the possibility of preventing intracranial hemorrhage at the partus. Prothrombin in the newborn child is lower than in the mother, and can be raised by giving vitamin K either to the mother before delivery or to the infant (97, 98). The moderately low prothrombin level in the first week of life is independent of the presence of icterus neonatorum. Very marked hypoprothrombinemia has been found in infants suffering from icterus gravis and some cases of related diseases without symptoms of occlusion of the bile duct. In some of these cases the prothrombin level was only a fraction of a per cent, while the prothrombin level of normal infants did not fall below 10 per cent. Vitamin-K treatment was effective in these cases as well as against the physiologic hypoprothrombinemia (63). As in the cases reported by Waddell *et al.* the "physiologic" reduction of the prothrombin was found to disappear at the end of the first week without treatment. Nygaard (64) examined independently fifty-four newborn babies by the method of Quick, using his own photoelectric apparatus for registering the coagulation time (71). The results were in accordance with those already described. The longest prothrombin times were found in infants with hemorrhages. In nine infants treated intramuscularly with vitamin K, no increase in prothrombin time occurred. Three infants with manifest hemorrhagic hypoprothrombinemia were successfully treated, intramuscularly and perorally, with a large quantity, 100,000 Dam units of vitamin K in all.

Alimentary vitamin-K avitaminosis in man.—The lack of vitamin K in the alimentary canal of man was observed and treated with vitamin K by Kark & Lozner (93). They used the complete method of Quick (32), in which the prothrombin time is determined not only on the plasma itself, but also on mixtures of the plasma with prothrombin-free plasma. By this procedure it is possible to draw conclusions regarding the prothrombin content.

The failure of vitamin K to influence the abnormal coagulation in hemophilia and thrombopenia has been stressed (51, 65). These ob-

servations are in accord with previous findings of the Copenhagen workers (44, 76). Cheney (85) reported that while most of his cases of hemophilia did not respond to vitamin K, one exceptional case responded slowly. This observation is in disagreement with those of the other investigators and must be considered with reservation.

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THE WATER-SOLUBLE VITAMINS¹

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THIAMIN (VITAMIN B₁)

Methods for the determination of thiamin and phosphorylated thiamin (cocarboxylase).—Prebluda & McCollum (1) introduced a new reagent, *p*-aminoacetophenone, for the quantitative determination of thiamin. The details of this method for thiamin and cocarboxylase were carefully worked out for biological materials by Melnick & Field (2, 3, 4). The thiochrome method of Jansen (5) has been modified by Hennessy & Cerecedo (6), Mukherji (7), and Wang & Harris (8). The rat growth method (9, 10), bradycardia (12), and pigeon curative method (11) all received considerable attention. The yeast fermentation technique (13) was developed as a micromethod.

Silverman & Werkman (67) described a technique for the assay of thiamin based on the increased anaerobic metabolism of pyruvate on the addition of thiamin to deficient cells of *Propionibacterium pentoseaceum*. Care must be taken, however, in the use of this method since these organisms can be "trained" to synthesize thiamin.

Distribution in foods.—Booher & Hartzler (15) reported the thiamin values of eighty-two foods, assayed by the rat growth method. The data represent an important contribution to our knowledge of the distribution of thiamin in foods since all of the usual foods were assayed in the same laboratory by the same method with an estimated precision of 15 to 20 per cent. Lunde *et al.* (68) studied the thiamin content of Norwegian wheat, rye, and flour.

Schlutz & Knott (16) studied the losses of thiamin in the preparation of evaporated milk, and Mickelsen *et al.* (69) studied the loss of thiamin in meats during frying, roasting, boiling, and stewing. The latter also determined the thiamin content of a large number of meats and meat products.

¹ The literature on the water-soluble vitamins is so enormous that it is quite impossible to cover all of it in the space allotted to this review. Much valuable material has been omitted because of limitation of space. The help of Dr. Marianne Frank in covering the German clinical literature is gratefully acknowledged.

The thiamin content of elephant's milk (54) was found to be about 25 international units per 100 cc., or about the same as good cow's milk. Guinea pig's milk (55) was also found to contain about 20 international units per 100 cc., but rat's milk was definitely richer, containing 50 to 80 international units per 100 cc. "Royal jelly" (73) was found to contain 1.0 to 1.5 international units of thiamin per gram.

Distribution in soil.—Lilly & Leonian (17) studied the distribution of thiamin in soil and found the greatest concentration in the first foot of soil. The soil zeolites hold the thiamin and, presumably, the roots find no difficulty in eluting it.

The "sparing" action of fat on thiamin.—There is now common acceptance of the idea (18) that fat "spares" vitamin B₁. Additional confirmation has come from Stirn *et al.* (19), Melnick & Field (20), and Arnold (21). Arnold & Elvehjem (22) made the important observation that, regardless of the amount of fat in the diet, the thiamin required by the dog is related directly to the nonfat calories of the diet. Thus, dogs fed diets low in fat required 75 µg. of thiamin per 100 gm. of food. An increase in the fat content of the diet to 56.5 per cent decreased the thiamin requirement to 27.5 µg. per 100 gm. of diet. This level of thiamin, when calculated on the basis of the nonfat calories, was found to be the same as in the low fat diet, namely 75 µg. per 100 gm. of nonfat diet.

Of interest in this connection is the report of Richter & Barelare (74) who found that rats fed a synthetic diet without the vitamin-B complex, and allowed to choose their food, ate large amounts of fat but very little sugar. When fed thiamin, the rats chose sugar preferentially to the fat.

The significance of the sparing action of fat on thiamin could not at first be properly appreciated. The role of thiamin in the oxidation of glucose renders the phenomenon intelligible, since thiamin is needed for the oxidation of carbohydrates, but not for the oxidation of fats. This observation assumes special significance if we accept the ability of brain tissue, especially the cerebral cortex, to oxidize only carbohydrates (23). The brain would therefore be especially dependent on thiamin for its metabolism.

Coccarboxylase.—Final proof that the active form of thiamin in pyruvic acid oxidation is the pyrophosphate, coccarboxylase, was presented by Banga *et al.* (24). It was first necessary to explain why Peters (25) found thiamin more active in the "catatorulin" test than

thiamin pyrophosphate. This was done in a satisfactory way by showing that with brain *brei* or slices, thiamin pyrophosphate failed to reach the active centers, and was less active than thiamin. With finely ground brain, however, thiamin pyrophosphate was more active than thiamin. The whole problem was thus found to be a matter of permeability. The thiamin was active only after conversion to the pyrophosphate.

Goodhart & Sinclair (14) found that all of the blood cocarboxylase was within the cells. These investigators presented evidence that nucleated blood cells can phosphorylate thiamin, and they believe this to be a function common to all nucleated animal cells. The nucleated blood cells originating in the bone marrow are also active in this respect. The circulating form of thiamin, according to these investigators, is free thiamin or its monophosphate ester, but not cocarboxylase; neither blood plasma nor cerebrospinal fluid contained any cocarboxylase. The liver probably phosphorylates thiamin for purposes of storage, and hydrolyzes the product when it is necessary to replenish the blood thiamin. In the kidney also phosphorylation goes on; the process is probably essential for the reabsorption of thiamin in the tubules. No cocarboxylase was found in the urine. The kidney is also capable of dephosphorylation, since Melnick & Field (26) virtually flooded the organism (human) with thiamin monophosphate without any phosphorylated thiamin appearing in the urine.

Stirn *et al.* (19) made the interesting observation that rats on high fat, low thiamin diets grew well and had normal estrus cycles, but the content of cocarboxylase in the livers of these rats was as low as in the livers of polyneuritic rats. The brain tissue of rats, however, retained a greater reserve of cocarboxylase.

Ochoa (27) studied the synthesis *in vitro* of cocarboxylase from thiamin in liver, brain, muscle, and intestine. Of these tissues, only the liver showed a good capacity to phosphorylate added thiamin, probably according to the following equation: thiamin diphosphate \rightleftharpoons thiamin + phosphate. Energy is required to shift the equilibrium to the left. Little synthesis of cocarboxylase took place in the absence of oxygen. The synthesis reached a maximum represented by the cocarboxylase content of normal tissue. The synthesis was inhibited by iodoacetate, suggesting that the esterification of the thiamin with inorganic phosphate is coupled with the oxidation of triosephosphate.

Barron & Lyman (28) studied the metabolism of pyruvic acid by gonococci, hemolytic streptococci, and staphylococci, and found that

it may go in two directions: (a) under optimal conditions of oxidation the pyruvic acid may be directly oxidized to acetic acid and carbon dioxide; (b) under optimal conditions for reduction it may be reduced to lactic acid or it may be split by dismutation into acetic acid and formic acid. Cocarboxylase acts as the catalyst for both the oxidation and dismutation of pyruvic acid.

Pyruvic acid in thiamin deficiency.—Lu (29) used the color of the 2,4-dinitrophenylhydrazone of pyruvic acid to develop a rapid, specific, and sensitive method for determining small amounts of pyruvic acid in biological fluids. In the rat suffering from thiamin deficiency (30) the concentration of blood pyruvate paralleled the degree of bradycardia. Intravenous injection of pyruvate into normal rats and rabbits, even above the highest levels occurring in thiamin deficiency, had no effect on the heart.

Banerji & Harris (31) studied the excretion of bisulfite-binding substances in normal and thiamin-deficient rats. They found that the excretion of bisulfite-binding substances increased with increasing degrees of deficiency.

Bollman & Flock (32) studied the pyruvate content of working muscles in normal and thiamin-deficient rats. Though the blood and muscles of thiamin-deficient rats contained more pyruvate than those of normal rats, the increase of pyruvate in contracting muscle was of the same order in both normal and deficient rats.

Lu & Platt (33) studied the effect of light muscular exercise on thiamin-deficient human subjects. The blood pyruvate was increased and the increment was often maintained or still further increased at the end of half an hour of rest after the exercise. Values for blood pyruvate as high as those found in fulminating beriberi were found in deficient subjects after exercise. In normal subjects after half an hour of rest the blood pyruvate returned to normal.

The pyruvic acid does not account for all of the bisulfite-binding substances in the human blood or urine. Simola (34) found an increase in the excretion of keto acids in rats on diets deficient in the vitamin-B complex. The increase in keto acid excretion could be largely accounted for by an increased excretion of α -ketoglutaric acid. Autoclaved yeast alone did not lower the excretion of the keto acids, but there was a marked lowering on the administration of thiamin. In beriberi (35), the increase in bisulfite-binding substances was greater than could be accounted for by pyruvic acid. Pyruvic acid values of the blood could often be restored to normal while at the

same time there was only a slight reduction in the bisulfite-binding substances.

Possible untoward effects of excess thiamin.—Sure (36) and Perla & Sandberg (37) observed that in rats on stock diets daily doses of 100 µg. or more of thiamin resulted in disturbances in lactation and reproduction. The daily addition of 2 mg. of manganese per rat completely prevented the toxic manifestations of excess thiamin. Supplementation with 2 mg. of manganese alone per rat per day also resulted in interference with lactation, particularly after one generation.

Engel & Phillips (38) found that the administration of thiamin to thiamin-deficient rats and chicks resulted in abnormal livers characterized by hydropic degeneration and excessive production of free fat in the liver cells; this resulted in disruption of the normal cell structure. Desiccated thyroid prevented these abnormal liver changes and starvation reduced their severity. Choline, casein, or diets high in fat did not prevent these changes. The rats with abnormal livers made remarkable gains in weight and showed no other ill effects.

Steinberg (39) used large doses of thiamin to treat cases of chronic arthritis. About three out of three hundred cases developed herpes zoster. Goodman (40) obtained favorable results by treating herpes zoster with thiamin and Rattner & Roll (41) obtained inconclusive results.

Lehmann & Nielsen (42) treated a beriberi patient with thiamin and after two months' treatment, the patient succumbed to pellagra. The writers explained the occurrence of pellagra as evidence of a disturbance of balance between the members of the vitamin-B complex.

Physiology.—Additional work on the relation of thiamin to the thyroid was reported by Peters & Rossiter (43) who found that hyperthyroidism caused a fall in tissue cocarboxylase and free thiamin. It was quite obvious that excess thyroxin increased the need for thiamin. Such data have been put to clinical advantage by Frazier & Ravdin (44) who treated hyperthyroid patients preoperatively with thiamin and brewers' yeast. The treatment shortened the period required for adequate preoperative preparation. They suggest that thiamin deficiency is likely to exist along with hyperthyroidism and is partly responsible for some of the symptoms, such as tachycardia.

Briem (45) found that thiamin increased the action of acetylcholine. This can be explained by the observation of Glick & Antopol

(46) that cholinesterase was inhibited by thiamin, thereby slowing down the hydrolysis of the acetylcholine.

Kuhn *et al.* (70) reported that acetylated thiamin acted on intestinal strips in a way similar to acetylcholine. Thiamin itself was inactive.

Molnár & Petrányi (47) described an interesting case of trigeminal neuralgia characterized by an inability of the patient to utilize injected thiamin, most of which was excreted in the urine. When cortin was injected along with the thiamin, the patient was able to retain a large part of the injected thiamin and was likewise cured of the trigeminal neuralgia. The author was led to this trial by Laszt's observation (48) that thiamin produced its effect only in the presence of adrenocortical hormone and vice versa.

During ether narcosis, there seems to be a tendency for the glycogen content of the liver to fall. This drop in liver glycogen was decreased by thiamin administration for three days previous to the narcosis (49). Thiamin administration immediately before the narcosis was without effect.

The mammary gland appears to offer much resistance to the transfer of thiamin from the food to the milk. Morgan & Haynes (50) found that the thiamin content of human milk was raised by thiamin administration only when milk of low thiamin content was secreted. Thiamin administration to a woman secreting milk of a high thiamin content did not further raise the thiamin content of the milk. There seems to be a maximum level beyond which the thiamin content of the milk of either humans or cows cannot be raised. This level appears to be 25 to 32 μ g. per 100 gm. of milk. In the rat also (55) it was impossible to increase the thiamin content of the milk by feeding large quantities of thiamin. However, there was a sharp drop in the thiamin content of rat's milk when a thiamin deficient diet was fed.

Of importance is the report of Naide (72) who relieved rest pain of ischemic origin with thiamin. To do so, the enormous dose of 100 mg. was used, and it was necessary to administer it intravenously. Apparently, a local avitaminosis resulted as a consequence of poor circulation, and a high concentration of thiamin in the blood was essential to supply it adequately to tissues with a reduced blood supply.

Experimental thiamin deficiency in the human.—Experimental thiamin deficiency in the human has been induced by several investi-

gators (51, 52, 53). Deficiency symptoms appeared in three to seven weeks. The symptoms observed were fatigue, lassitude, anorexia, precordial pain, burning of the feet, dyspnea on exertion, muscle cramps, palpitation, constipation, loss of weight, and absence of or low gastric acidity (free). The objective symptoms were skin hyperesthesia in a sock distribution and changes in the electrocardiogram. Abnormality in electrocardiograms developed. The blood bisulfite-binding substances were not abnormal. Recovery promptly followed the administration of thiamin.

Methods for assessing the level of nutrition.—Melnick *et al.* (51) have established 90 μ g. for the male and 60 μ g. for the female as the minimum normal values of thiamin in a twenty-four-hour sample of urine. Values below these they considered to be evidence of a possible thiamin deficiency. To confirm this suspicion, a test dose was administered orally with a meal. If 10 to 20 per cent of the test dose (5.0 mg.) was excreted, there was considered to be no thiamin deficiency. If 7 to 10 per cent of the test dose was excreted, the subject was mildly deficient. If 1 to 7 per cent of the test dose was excreted, the subject was markedly deficient. The test dose must be given with the meal because when given on an empty stomach, there is considerable destruction of thiamin before absorption. Administering the test dose with the meal was found preferable to parenteral administration, since the flooding of the organism with thiamin had a tendency to mask existing thiamin deficiency.

Schroeder (56) studied the effect of administering 5 to 10 mg. of thiamin to patients with cardiac insufficiency, gastritis, nephrosis, and Parkinson's disease. The thiamin excretion was irregular, but generally below that obtained in the normal human. The presence of hemin and, to a lesser degree, the presence of bile in the gastric fluids was destructive to thiamin.

Drigalski (57) found on continuous daily injection of 10 mg. of thiamin that there was great destruction of the vitamin. Similar results were obtained by Sciclounoff (58) who injected 50 mg. of thiamin daily and generally recovered but 12 to 20 per cent in the urine, most of the rest presumably being destroyed. Pregnant women (59) were found to excrete subnormal amounts of thiamin; one exception was found—a woman who always ate whole wheat bread.

Widenbauer & Wieland (60) used the urinary excretion of thiamin in a new way to determine the thiamin requirements of nursing babies and adults. A low thiamin ration was fed until no urinary

thiamin was excreted and there was a reduction in the thiamin of the blood. Thiamin was added until it was excreted in the urine again and the blood thiamin returned to its normal values. By this "back-titration" they found that the thiamin consumed per 100 cal. was 16 μ g. This figure is below the usual standards.

Clinical.—Disturbances of the circulation in alcohol addicts respond favorably to thiamin therapy (61) provided the cardiovascular disturbances are recent. Additional work on the effects of thiamin in alcoholic neuritis was published by McGee (62). Favorable results with thiamin were obtained in the treatment of nonanginous precordial pain (63), neuralgia of amputation stump (64), and nervous diseases (71). Sinclair (66) reviewed the causes of thiamin deficiency.

RIBOFLAVIN (VITAMIN B₂)

Methods.—Sullivan & Norris (78) studied the chemical determination of riboflavin in milk products. A fluorometric method (79) has been proposed by Supplee *et al.* Reindel & Fleischmann (80) determined riboflavin by converting it to lumiflavin and extracting it with chloroform.

Lunde *et al.* (81) measured the color of the riboflavin after destroying impurities by oxidation with potassium permanganate. By this method they obtained values which agreed well with biological tests. Snell & Strong (82) have worked out a microbiological method for determining riboflavin by taking advantage of the indispensability of riboflavin for *Lactobacillus casei*.

Distribution.—The riboflavin in meat and meat products was studied by Mickelsen *et al.* (83) by the bacteriological method (82). Lunde (84) studied the distribution of riboflavin in fish and fish products. Fish roe and fish liver were especially rich sources of riboflavin. Little destruction took place on preservation (87).

Markuze (108) examined elephant's milk for its riboflavin content and found by direct comparison that it contained about as much riboflavin as cow's milk.

The riboflavin content of the milk of rats and guinea pigs (85) was determined and compared with cow's milk. Rat's milk was found definitely richer and guinea pig's milk poorer than cow's milk. The riboflavin of rat's milk could be increased by feeding lactating rats large quantities of riboflavin.

Lunde *et al.* (86) determined the distribution of riboflavin in Norwegian flours and breads.

Role in rats, dogs, pigs, chickens, and man.—Nerve degeneration,

which takes place in chicks fed a low riboflavin diet (88), did not occur in rats fed a similar ration (89).

The importance of riboflavin for the eye has been emphasized by Day *et al.* (90), who first showed it could prevent cataract in rats. Kimble & Gordon (91) observed that individuals who show low biophotometer readings do not always respond to vitamin-A administration. Those failing to so respond, responded with normal biophotometer readings immediately when both vitamin A and riboflavin were administered. This observation becomes additionally significant in the light of Pock-Steen's observation (92) that "twilight blindness," frequently occurring in patients with sprue, can be cured by doses of 1 mg. of riboflavin. In these cases vitamin A is without effect and it is, therefore, different from night blindness. Certain other eye abnormalities responded to riboflavin therapy.

Sebrell & Butler (109) produced riboflavin deficiency in thirteen out of eighteen women fed a low riboflavin diet. They developed reddened, denuded lesions of the lips, maceration and fissuring in the angles of the mouth, and seborrheic accumulations at the nasolabial folds. These lesions disappeared following the administration of riboflavin and reappeared after the discontinuance of the riboflavin. One woman who did not receive any riboflavin showed no lesions at any time during the 365 days of observation. These investigators, while recognizing the therapeutic value of crystalline vitamin preparations, emphasized their limitations in the presence of multiple deficiencies such as are usually encountered.

Street & Cowgill (93) developed acute riboflavin deficiency in the dog. They confirmed Sebrell & Onstott's finding (94) that dogs on a purified diet lacking riboflavin suddenly collapsed. During periods of collapse there was a marked fall in body temperature and respiration rate. In five out of seven dogs recovery followed the injection of 0.75 mg. of riboflavin per kg. in from three to twenty-four hours. Twenty-five μ g. of riboflavin per kg. were suggested as sufficient to satisfy the riboflavin requirements of the dog. These investigators suggested as an explanation for this collapse the role of riboflavin in the enzyme system for the oxidation of carbohydrates. This becomes especially significant since sugars are the chief if not the only fuel which the brain can utilize as its source of energy (95).

Hughes (96) studied riboflavin deficiency in the pig. The deficient pigs grew slowly and became crippled. The crippled condition was not corrected on feeding riboflavin, thus suggesting an irreparable nerve damage.

Riboflavin seems to be especially important for the laying hen (97, 98). Hunt *et al.* (97) and Lepkovsky *et al.* (75) found that riboflavin increases egg production, but Davis *et al.* (76) disagree. The difference may be due to the breed of the hens, riboflavin being more important for egg production in some breeds than in others.

There was no disagreement, however, on the importance of riboflavin for hatchability. Hunt *et al.* (97) found that 220 to 230 μg . of riboflavin per 100 gm. of feed are required for good hatchability and this agrees closely with the results of Schumacher *et al.* (98) who found 230 μg . per 100 gm. of feed adequate. The riboflavin of the feed definitely influenced the riboflavin content of the eggs (75, 97) which in turn influenced the livability of chicks on riboflavin-deficient diets; this shows that the riboflavin content of the egg is important in determining the store of riboflavin in the newborn chick.

When riboflavin in the feed was inadequate for hatchability the embryonic mortality proved to be more or less characteristic. Schumacher *et al.* (98) found retarded growth of the embryos, curled toes, incomplete yolk absorption, scarcity of down and, in some cases, clubbed down. Apparently, embryonic abnormalities need not always be uniform since characteristic abnormalities in California (75) were found to be retarded growth, clubbed down, degeneration of the Wolffian bodies, edema, and anemia.

Role in metabolism.—Chevremont & Comhaire (99) studied the effect of cyclopentylidinitrophenol, which accelerates tissue metabolism, upon the free and combined riboflavin in the liver, kidney, muscle, heart, and lung. There was an increase of free riboflavin in all of the tissues with the smallest increase being found in the heart. There was a decrease of combined riboflavin in all of the tissues except the liver, in which there was an increase. Presumably the free riboflavin liberated in the organs accumulated in the liver, partly free and partly combined.

Robbers & Westenhoeffer (100) studied the effect of riboflavin and corticosterone on the phlorhizin glycosuria experimentally induced in man. Neither riboflavin nor corticosterone nor riboflavin phosphate showed any influence on the glycosuria. The experiment was intended to throw light on the idea that phlorhizin glycosuria results from inhibition by phlorhizin of the phosphorylation of glucose (101) thus allowing glucose to escape into the urine.

Hirt & Wimmer (102) used the fluorescence microscope to study the fate of injected riboflavin in mice. With a large intravenous ad-

ministration of riboflavin only a small quantity was taken up by the cells, the largest portion being excreted into the intestinal canal by way of the bile capillaries. When given by mouth there was very little excretion by way of the bile. A single intravenous injection of riboflavin takes the following course. In two and one-half minutes there is a bright green fluorescence in the blood serum. In two and three-quarters minutes the fluorescence is present in the liver. In ten minutes the fluorescence in the blood is decreasing, but is now present in the liver and the bile capillaries. In one hour and fifteen minutes the fluorescence in the blood stream has disappeared but is still present in the liver and bile capillaries. After six and one-half hours the fluorescence has disappeared from the bile capillaries but is still present in the liver. It is to be noted how rapidly the tissues remove the riboflavin from the blood stream, the fluorescence of the blood decreasing in ten minutes.

Riboflavin complexes, enzymes, prosthetic groups.—Schormuller (103) studied the combination of riboflavin with various proteins. His studies included the adsorption of riboflavin from solutions containing variable amounts of riboflavin with the same amount of protein and with variable amounts of protein with the same amount of riboflavin. All variables affected the adsorption of riboflavin by the protein.

The alloxazine-adenine-dinucleotide has been extensively studied. Adler and associates (104) pointed out that there are two diaphorases, diaphorase I which is specific for dihydrocodehydrase I and diaphorase II for dihydrocodehydrase II. Lockhart (105) found that diaphorase is present in the tissues of some of the higher plants and absent or nearly so from those of other higher plants and fungi. Plant diaphorase was found to be specific for dihydrocodehydrase I and extremely unstable.

Axelrod *et al.* (106) studied the effect of riboflavin deficiency on the *d*-amino acid oxidase content of rat's liver and kidney. The livers of deficient rats showed marked decreases in both riboflavin and *d*-amino acid oxidase. Slight decreases in riboflavin and *d*-amino acid oxidase were observed in the kidney. Riboflavin supplementation restored the riboflavin content of the liver to normal but raised the *d*-amino acid oxidase content only to 50 per cent of its normal value. The *d*-amino acid oxidase of the liver was restored to normal by adding a butanol extract of a liver concentrate in addition to the riboflavin supplement. Riboflavin alone was sufficient, however, to

restore to normal both the riboflavin and *d*-amino acid oxidase of the kidney.

Riboflavin seems also to take part in the pyruvic acid oxidation system. Lipman (107) obtained a protein fraction from lactic acid bacteria which on addition of thiamin pyrophosphate did not catalyse the oxidation of pyruvic acid, but did so on addition of both thiamin pyrophosphate and flavin-adenine-dinucleotide.

NICOTINIC ACID

Methods.—Chemical methods for determining nicotinic acid have been studied by Pearson (110), Bandier & Hald (111), Bandier (112), Kringstad & Naess (113), and Ritsert (119). The methods are based on the color produced when pyridine reacts with cyanogen bromide and a primary or secondary aromatic amine. In these studies, aniline (110, 113, 119) and metol (*p*-methylaminophenol) (111, 112) were used.

Dorfman *et al.* (114) used the dysentery bacillus for the quantitative determination of nicotinic acid. Insects were suggested (115) as test organisms capable of detecting 1 μ g. of nicotinic acid.

Margolis *et al.* (116) improved the blacktongue-producing diet by correcting secondary deficiencies of thiamin and riboflavin, thus making the test for nicotinic acid with the dog more specific.

Axelrod & Elvehjem (117) studied the fermentation method of Euler (118) for determining coenzyme I and described in detail their procedure.

Distribution.—Bandier (112) studied the distribution of nicotinic acid in the muscles and a large number of organs of the pig and the ox. The liver was richest, containing about 12 mg. per 100 gm. of fresh tissue.

Dorfman *et al.* (114), using the dysentery organism, determined the nicotinic acid content of human blood and urine, milk and saliva. Almost all the activity of the blood was found in the erythrocytes. Pearson (110), using a chemical method, also found that the nicotinic acid existed largely in the erythrocytes of the blood of the dog, pig, sheep, and horse.

Kringstad & Naess (113) assayed a large number of foods for nicotinic acid. Bandier & Hald (111) reported the nicotinic acid content of various yeasts with results varying from 16 to 60 mg. per 100 gm. of dry yeast. Surface yeasts were more potent than bottom yeasts. Wine yeasts were poorest.

Axelrod & Elvehjem (117) determined the coenzyme-I content of

a number of normal tissues of guinea pigs, rats, chickens, and dogs. The liver, kidney cortex, and muscle were richest.

Euler & Schlenk (125) determined the cozymase content in the blood of the rat, rabbit, cow, horse, and pigeon, and found values ranging from 50 to 200 $\mu\text{g. per cc.}$ Human blood contained 40 to 80 $\mu\text{g. per cc.}$ The serum contained practically no cozymase. The cozymase in the muscle, heart, kidney, brain, liver, and the sciatic nerve of the rat varied from 150 to 200 $\mu\text{g. per gm. of fresh tissue.}$ The ratio of cozymase to dihydrocozymase ($\text{Co}:\text{CoH}_2$) in most of these organs was about 3:1.

In the muscle and in most organs the amount of codehydrogenase II is small compared with cozymase I, but in red blood cells the codehydrogenase II is higher.

The nicotinic acid amide found in the blood of the cow, rabbit, and rat varied from 2 to 3 $\mu\text{g. per cc.}$ In the human about 1.5 $\mu\text{g. per cc.}$ were found. Since about 11 $\mu\text{g.}$ of nicotinic acid in human blood were present as codehydrase I and II (60 $\mu\text{g.}$), about one eighth of the total blood nicotinic acid exists in the free form.

Physiology.—The effect of a low nicotinic acid diet on the coenzyme-I content of the tissues of the dog and pig was studied by Axelrod *et al.* (120). The coenzyme content of the liver and muscle was lowered but no effect was noted on the coenzyme content of the brain, kidney cortex, and blood.

Kohn *et al.* (121) studied the coenzyme-like substances (V-factor) of the tissues of normal dogs, dogs with acute blacktongue, and dogs in normal health immediately after cure of blacktongue by small doses of nicotinic acid. There was a lowering of coenzyme-like substances in the liver (70 per cent) and muscle (35 per cent) with no change in the other tissues.

Kohn & Klein (122) and Vilter *et al.* (148) demonstrated the synthesis *in vitro* of the V-factor and cozymase I by human erythrocytes from nicotinic acid. The latter suggest that the nucleated cells are essential for the synthesis of coenzymes I and II from nicotinic acid.

Vilter *et al.* (123) using *B. influenzae* as the test organism (124) found the blood of pellagrins had a lower codehydrogenase content than the blood of normals. Feeding nicotinic acid restored the blood codehydrogenase to normal. Pellagra differs, therefore, from blacktongue since there is no lowered codehydrogenase content of the blood of the dog with blacktongue (120, 121).

The function of nicotinic acid.—Funk & Funk (126) discussed

the role of nicotinic acid on the food intake and water metabolism of rats and pigeons. Though animals receiving nicotinic acid consumed more food, they did not show the expected increase in weight over the controls not getting the nicotinic acid. This was due to retention of water by the nicotinic acid deficient animals.

Dorfman *et al.* (127) studied twenty-four compounds structurally related to nicotinic acid on the growth of the dysentery bacillus. The amide was about ten times as potent as the nicotinic acid. Any change in ring substitution resulted in loss of activity. Esterification resulted in a decrease of activity which proceeded in parallel with increase in length of the alkyl chain. Substitution on the amide nitrogen resulted in loss of activity. Nicotinuric acid was comparable in activity to nicotinic acid.

Madison *et al.* (128) reported an outbreak of nicotinic acid deficiency in swine in the field. Pure nicotinic acid stopped the heavy mortality and restored the remaining swine to normal health. Lambs on a pellagra-producing diet (129) developed normally. Birch (130) determined that 0.25 mg. of nicotinic acid per kg. allowed dogs to increase in weight rapidly, while 0.13 mg. per kg. prevented black-tongue and allowed a slight increase in weight.

Burke & McIntyre (131) found that nicotinic acid markedly decreased the duration of hypoglycemia following the injection of a standard dose of insulin in rats rendered hypersensitive by deficiency in the vitamin-B complex (other than thiamin). Mainzer (132) describes a hypersensitivity to insulin in pellagrins, characterized by an unusually large fall in the blood sugar and a very slow or no rise in blood sugar during a period of five hours thereafter. This sharp drop in blood sugar could be brought about by five units of insulin.

Nicotinic acid effected a remarkable improvement in stuporous patients (133) and in patients with sprue (134). A case of sprue following gastroenterostomy was described by Siedek & Reuss (135). The chief symptoms were diarrhea, disturbance of fat absorption, and a macrocytic anemia. Nicotinic acid improved fat absorption, cleared up the diarrhea, and increased the number of red blood cells, at the same time decreasing the color index from 2.3 to 1.04.

Crandall *et al.* (147) showed that gastrointestinal motility was very favorably influenced by nicotinic acid. Thiamin and riboflavin seemed to be without effect.

The urinary pigments in alcoholic pellagra were investigated by Watson (149). He found that the Beckh, Ellinger & Spies test (150)

for the detection of porphyrin in the urine of pellagrins was not specific for porphyrin, but included largely a red pigment which he isolated in crystalline form. The chemical nature of the pigment remains to be established. Nicotinic acid therapy decreased the urinary content of the red pigment.

The pellagra problem.—While nicotinic acid will cure most of the usual symptoms of pellagra, it apparently cannot solve the pellagra problem. Practically never have uncomplicated nicotinic acid deficiencies been encountered. Thiamin and riboflavin deficiencies accompanied nicotinic acid deficiency in most pellagrins (123, 136, 137, 138). In these communications the importance of feeding pellagrins a well-balanced diet with nicotinic acid was emphasized.

Spies *et al.* (139) frequently encountered pellagra in infancy and childhood and found it usually associated with inadequate maternal diets during pregnancy and lactation. In such cases, lesions characteristic of pellagra were seldom found. In the absence of typical lesions, the administration of one of the therapeutic agents specific for pellagra offered a valuable therapeutic test in confirming the diagnosis of latent pellagra.

Two new specific remedies for the cure of pellagra have been discovered, pyrazine-2,3-dicarboxylic acid (140) and quinolinic acid (141). Since quinolinic acid (142) was ineffective in curing black-tongue in dogs, it must be accepted as indicative of a difference between black-tongue in dogs and pellagra in human beings. Moreover, as a result of the administration of quinolinic acid there was an increase in the concentration of coenzymes I and II in the blood. Such an increase fails to take place in the dog even when nicotinic acid is administered (120, 121).

Mainzer reported an interesting case of pellagra in identical twins (143) who had the same type of pellagra. The disorder, as well as four relapses and remissions, developed simultaneously in the two sisters.

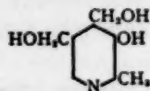
Sydenstricker *et al.* (144) showed that whatever factor or factors cure pellagra, they were absent from the liver of a pellagrin, but were present in normal livers. Studies were reported on pellagra in Great Britain since 1934 (145) and in Kentucky mountain folk (146).

PYRIDOXIN (VITAMIN B₆)

[Factor 1 (169), rat antidermatitis vitamin, antiacrodynia vitamin, adermin (170)]

Structure and synthesis.—With the isolation of vitamin B₆ (151) there followed almost immediately communications on the chemical

structure of the vitamin. Kuhn *et al.* in a series of publications (152 to 156) showed that the formula for vitamin B₆ is



This vitamin B₆ is a pyridine derivative so that with nicotinic acid, two vitamins of the B complex are pyridine derivatives.

Independent confirmation for this structure was quickly forthcoming from the work of Keresztesy *et al.* (157), Stiller *et al.* (158), and Harris *et al.* (159). Almost at the same time the synthesis of vitamin B₆ was reported (160, 161). An improved method for the synthesis (162) was later published by these workers. The synthesis of vitamin B₆ was also accomplished by Kuhn *et al.* (163). Ichiba & Michi (164, 165, 166) have also made important contributions to our knowledge of the chemical structure of vitamin B₆. These investigators (164) pointed out that as far back as 1931 Ohdahke, working with Suzuki, had isolated a crude preparation of vitamin B₆ from which pure vitamin B₆ was readily obtained. Ohdahke was ahead of his time since the technique for the biological recognition of vitamin B₆ had not yet been developed.

Nomenclature.—The term factor Y was first used by Chick & Copping (167) for the factor which György (168) later called vitamin B₆. The term vitamin B₆ has been replaced in a limited way by factor 1 (169) and adermin (170). The vitamin has also been commonly referred to as the antiacrodynia vitamin and the rat antidermatitis vitamin. The nomenclature was additionally complicated by the inability of Dann (171) to regularly produce dermatitis in rats in the absence of the rat antidermatitis vitamin. On the other hand György & Eckhardt (172) produced dermatitis in rats receiving the antidermatitis vitamin, though apparently the dermatitis was different from the "specific dermatitis" (168) referable to vitamin B₆. The determination of the chemical structure of vitamin B₆ opened the way for clearing up this confusion by adopting a name descriptive of the chemical compound, 2-methyl-3-hydroxy-4,5-di-(hydroxymethyl)-pyridine (vitamin B₆). György & Eckhardt (172) suggested pyridoxin as sufficiently characterizing the compound so that confusion could be avoided in the future. The term pyridoxin will henceforth be used to replace vitamin B₆, factor 1, adermin, rat antidermatitis vitamin, and the antiacrodynia factor.

Physiology.—Fouts *et al.* (173) in continuing the work on the microcytic hypochromic anemia in dogs deficient in pyridoxin showed that the anemia could be cured by the administration of crystalline pyridoxin in quantities of 60 $\mu\text{g.}$ per kg., thus proving that there is an intimate relationship between pyridoxin and the formation of hemoglobin.

A similar observation in the pig was reported by Chick *et al.* (174). In the absence of pyridoxin the pigs developed a microcytic hypochromic anemia which was readily cured by pyridoxin fed as an eluate of a fuller's earth adsorbate prepared from liver. During the deficiency the pigs suffered from epileptic fits similar to those seen in the human being.

Dimick & Schreffler (175) fed graded amounts of pyridoxin to rats and regularly cured dermatitis with 5 $\mu\text{g.}$ daily. A level of 3 $\mu\text{g.}$ daily, while promoting considerable growth, cured dermatitis but irregularly, and 1 $\mu\text{g.}$ daily no longer was capable of curing dermatitis but still supported some growth. Rats receiving pyridoxin, but with their food intake limited to the amount eaten by pyridoxin-deficient rats with which they were paired, contained considerable body fat and were somewhat heavier than their paired controls. The pyridoxin deficient rats were devoid of fat thus indicating that pyridoxin enabled rats to utilize their food more efficiently.

The skin lesions in rats resulting from a combined deficiency of pyridoxin and the "rat growth factor" were studied histologically by Antopol & Unna (176). In this connection it might be noted that Quackenbush *et al.* (177) were able to cure similar lesions in rats by the administration of fats.

The antiacrodynic properties of fifty-two foodstuffs were quantitatively determined by Schneider *et al.* (191) and their activities were expressed as minimal amounts of material required to cure dermatitis (acrodynia). The basic diet did not include the "rat growth factor," and crystalline pyridoxin relieved the dermatitis (acrodynia) only temporarily. Fruits and vegetables were poor sources of the antiacrodynic factor; fish and meat were fair sources, while seeds, legumes, and cereals were relatively rich sources. Extremely potent were certain vegetable fats.

Lunde (190) reported the pyridoxin content of various fish and fish products. He found cod liver to be almost as rich as yeast or wheat germ and cod roe also a rich source. Sardines were fair sources and cod meat or milt were fairly poor sources.

Carter & O'Brien (178) showed that pyridoxin was essential for the chick and pigeon. The essential nature of pyridoxin for the chick found immediate confirmation (179, 180).

As might be expected, pyridoxin plays a role in human nutrition. Spies *et al.* (181) reported as symptoms of pyridoxin deficiency in the human, extreme nervousness, irritability, abdominal pain, weakness, and difficulty in walking. In such patients dramatic relief of symptoms and return of strength within four to twenty-four hours followed the administration of 50 mg. of synthetic pyridoxin. This dramatic clinical response is similar to the response obtained in rats.

Pyridoxin was shown to be essential for the growth of yeast (184, 185), *Streptobacterium plantarium* (182), and lactic acid bacteria (183). Robbins & Schmidt (186) showed that pyridoxin is needed by excised tomato roots.

Microorganisms seem to possess the ability to synthesize pyridoxin, as shown by McElroy & Goss (187). These workers determined the pyridoxin content of feed fed to sheep and then quantitatively determined the pyridoxin in the contents of the sheep's rumen and reticulum and found active synthesis of pyridoxin to have taken place.

Chemistry.—Greene (188) added a new and simplified method for the preparation of crystalline pyridoxin from natural sources. In addition to greater simplicity, the method seems to give greater yields of pyridoxin. Smith & Dimick (189) showed that the pyridoxin in a rice bran concentrate is stable to sulfur dioxide.

Kuhn & Löw (192) reported at some length their attempts to work out a colorimetric method for the quantitative determination of pyridoxin. The use of the Folin-Denis phenol reagent seemed promising. Since the colorimetric test for the determination of nicotinic acid is based on its character as a pyridine derivative, the possible interference of pyridoxin, also a pyridine derivative, with the determination of nicotinic acid was investigated. Pyridoxin did not interfere with the nicotinic acid determination.

THE UNIDENTIFIED MEMBER OR MEMBERS OF THE VITAMIN-B COMPLEX

[Filtrate factor (193), yeast or liver filtrate factor (194), factor 2 (195), factor W (196), chick antidermatitis vitamin (197), chick antidermatosis vitamin (198), the anti-grey hair factor (233), *filtrat wachstum faktor Bw* (199).]

Terminology.—When the term "filtrate factor" was first introduced (193) it was defined as "a water-soluble vitamin belonging to

the vitamin-B complex and preventing a dietary dermatitis in chicks." Thus it was limited strictly to chickens. Concentrates preventing and curing chick dermatitis also carried a factor required by rats (195), in addition to thiamin, riboflavin, and pyridoxin (vitamin B₆). To guard against possible nonidentity of the chick antidermatitis vitamin and the factor necessary for rats, the latter was provisionally called factor 2 (195).

The term "filtrate factor" has been widely adopted, but it is seldom used in the sense as originally defined. In fact its usage is almost limited to rats. For the sake of convenience, the factor preventing or curing a dietary dermatitis in the chick will be referred to as the chick antidermatitis vitamin. The factor necessary for rats will be referred to simply as the "rat growth factor" and will mean the unidentified factor or factors required by the rat in addition to thiamin, riboflavin, and pyridoxin (vitamin B₆).

Properties of the "rat growth factor."—Macrae *et al.* (194) have prepared a potent extract of the "rat growth factor" (liver filtrate factor) by the use of such reagents as amyl alcohol, lead acetate, mercuric acetate, and fuller's earth. With this concentrate they established the following properties for the "rat growth factor": It is not precipitated from aqueous solution by salts of lead, mercury, silver, quinine, or brucine; it is precipitated from alcoholic solution by barium hydroxide; it is extracted from acidified solution by amyl alcohol and ether; it is not adsorbed by fuller's earth but is adsorbed by large amounts of charcoal from which it can be eluted with 1 per cent aqueous sodium hydroxide; it is readily acetylated to form chloroform-soluble inactive acetyl derivatives whose activity can be regenerated by mild hydrolysis; esterification with diazomethane does not appreciably alter the activity of the "rat growth factor"; the factor is easily destroyed at 100° by 5 per cent sodium hydroxide or 10 per cent sulfuric acid. Treatment of their highly potent concentrates with alcohol and chloroform yielded 10 mg. of material with a "rat day dose" of 180 µg., the most potent concentrate of the "rat growth factor" thus far prepared.

Hitchings & Subbarow (200) concentrated the "rat growth factor" by the use of amyl alcohol, charcoal, brucine, chloroform, alcohol, and ether. Treatment with acid at 100° was less destructive to the crude ether extract than to the more highly purified "ether precipitate."

Kringstad & Lunde (199) studied the adsorbability of the "rat growth factor" (*filtrat wachstums faktor Bw*) with fuller's earth at

various pH concentrations, but found it unadsorbable at any pH. The factor was not precipitated by mercuric acetate nor could they extract it from a strongly acidulated yeast concentrate with ether.

Factor W (196) differs principally from the "rat growth factor" in that pyridoxin (vitamin B₆) is not included in the basic test diet, so that factor W must be considered as representing at least two factors, namely, pyridoxin and the "rat growth factor." Factor W could be adsorbed on norite, eluted, and further concentrated by the use of such solvents as butyl alcohol, ether, or phenol. It could not be acetylated and successfully hydrolyzed.

Properties of the chick antidermatitis vitamin.—Smith & Dimick (232) showed that the chick antidermatitis vitamin was stable to sulfur dioxide but was destroyed to varying degrees in both yeast and rice bran concentrates by autoclaving. Jukes (202) carefully studied the heated diet (201) for its completeness as a test diet for investigations of the chick antidermatitis vitamin. Substitution of sardine meal for casein did not improve the diet. The possibility of arginine deficiency (203) was examined and it was found that addition of arginine did not improve the diet.

By a study of the distribution of the chick antidermatitis vitamin between acetone and a whey concentrate at pH 3 to 8 (202) the activity was found to remain in the whey above pH 6 and to pass into the acetone below pH 6, from which it was concluded that the chick antidermatitis vitamin was an acid completely dissociated at pH 6. The vitamin would, therefore, be an acid with a dissociation constant between 2×10^{-6} and 5×10^{-6} .

The vitamin was destroyed by potassium permanganate, but not by manganous sulfate. Excess bromine in acid or alkaline solution did not destroy the vitamin. Neither did excess sodium hydroxide in the cold. Copper hydroxide followed by excess sodium hydroxide did not remove the vitamin nor did mercury or cadmium in alcohol with or without sodium hydroxide. In one case, however, mercury in alkaline ethanol did precipitate one half of the vitamin, the other half remaining in solution.

Possible relationship of the chick antidermatitis vitamin with a yeast factor, pantothenic acid.—The most spectacular advance in the elucidation of our knowledge of the chick antidermatitis vitamin was made by Woolley *et al.* (204) who showed that it was a complex of β -alanine and a hydroxy acid of unknown constitution. They showed this by inactivating a potent concentrate of the vitamin with heat in

acid or alkaline solution. The activity could be regenerated by coupling the acid chloride of the acetylated hydroxy acid with β -alanine ethyl ester and subsequently hydrolyzing off the ester and acetyl groups with cold sodium hydroxide. They suggested that the chick antidermatitis vitamin might be similar to pantothenic acid (205, 206) for the following reasons: (a) both are acids composed of β -alanine and a hydroxy acid in amide linkage; (b) both are labile to hot acid and alkali; (c) they form inactive acetyl derivatives which distill at approximately the same temperature and pressure; (d) the solubilities in various solvents of both of the free acids and their metallic salts are similar.

Jukes (207) supported the above contention by showing that a crude preparation of pantothenic acid obtained from R. J. Williams was potent in curing chick dermatitis, thus showing by direct test a similarity in properties of the chick antidermatitis vitamin and pantothenic acid. In a later communication Jukes (208) fed chicks a more highly potent concentrate of pantothenic acid, also obtained from R. J. Williams, and found that the chick antidermatitis vitamin was concentrated along with the pantothenic acid. He details the following properties as common to both factors: (a) resistance to chemical agents such as bromine, nitrous acid, and hydrogen peroxide; (b) lability to hot alkali, hot acid, and cold acid alcohol; (c) absence of amino, aldehyde, or ketone groups; (d) similar dissociation constants.

Contribution of knowledge obtained with microorganisms to the chemistry of the chick antidermatitis vitamin.—Whether or not pantothenic acid is identical with chick antidermatitis vitamin, a question which will be settled when either one is prepared in pure form, it is very definite that they have similar properties and behave similarly to chemical reagents. Since Williams' pantothenic acid represents the most potent preparation of the chick antidermatitis vitamin so far prepared, it is logical to use the methods for concentrating pantothenic acid (209) in studies on the chick antidermatitis vitamin. Hitchings & Subbarow (200) have already used parts of the procedure for concentrating the "rat growth factor."

Williams *et al.* (205) assigned the formula $C_8H_{14}O_5N$ to pantothenic acid. Weinstock *et al.* (206) showed that β -alanine was a cleavage product of pantothenic acid. It must, therefore, consist of β -alanine and a dihydroxyvaleric acid. The molecular weight was given as 205 (205), with a dissociation constant of 3.9×10^{-5} .

An important property of pantothenic acid is the inactivity of the

ester and acetyl derivatives (205). The activity of the ester has been regenerated by mild hydrolysis but no attempt seems to have been made to regenerate the activity of the acetyl derivative.

Snell, Strong & Peterson (210) found they could replace a factor for the lactic acid bacteria they were studying with Williams' pantothenic acid preparations and they concluded that their factor was identical with pantothenic acid. They could activate inactive preparations by coupling β -alanine with the remaining unknown acid by the method that Woolley *et al.* (204) applied successfully to the chick antidermatitis vitamin. Their preparation lost potency by heating at 100° at pH 10, but was stable at room temperature at pH 11. The active compound could be acetylated, esterified, or both acetylated and esterified. The acetylated ester could be concentrated by distillation which removed inactive material or material of low activity.

The anti-grey hair factor.—Morgan *et al.* (233) and Lunde & Kringstad (211) observed a greying of black rats on diets deficient in the "rat growth factor." They observed that greying could be accelerated by the addition to the diet of nicotinic acid or pyridoxin thus showing that other members of the vitamin-B complex influence greying. Lunde & Kringstad (211) claimed that the anti-grey hair factor was relatively unstable as compared with the "rat growth factor." Oleson *et al.* (212) presented data to show that the dietary factor which prevents greying (nutritional achromotrichia) is distinct from all factors of the vitamin-B complex necessary for the rat. Some slight activity was attributed to "pantothenic acid." Mohammad *et al.* (213) fed both an ether extract of molasses and the ether extracted residue and obtained equivalent growth with both extracts but the rats on the ether extract remained black while the rats on the ether extracted residue became grey. This indicated a greater relative solubility in ether of the anti-grey hair factor since, presumably, most of the anti-grey hair factor was extracted but much of the growth factor remained behind.

Morgan & Simms (214) have made the most thorough studies of this factor. They associated greying with coarse and lifeless fur and subnormal growth. Histologically, greying was associated with atrophy of the adrenals, loss of elastic layers of the skin, failure of spermatogenesis, and atrophy of the hair follicles. Injection of adrenocortical extract cured the greying but had no influence otherwise on the growth or well-being of the rat. These workers succeeded in producing greying of the hair in guinea pigs and Boston Bull pups.

Physiology.—Morgan & Simms (214) showed that absence of the "rat growth factor" brought about general changes of senescence such as greying of the hair, atrophic changes of the adrenals, testes, and skin. Lactation failed completely in the absence of the "rat growth factor." Occasionally large ulcers formed which cleared up only on addition of the "rat growth factor."

György & Goldblatt (216) reported that rats on basic rations lacking only the "rat growth factor" developed pathological changes in the liver. Yeast or yeast extract (Peters' Eluate) prevented this hepatic injury.

Daft & Sebrell (234) and Nelson (235) have studied hemorrhagic adrenal necrosis in rats receiving thiamin, riboflavin, and pyridoxin.

Phillips & Engel (215) found nerve degeneration of the spinal cord in 100 per cent of the chicks lacking the chick antidermatitis vitamin. The brain, apparently, was not affected. The degeneration could not be prevented by vitamin B₁, riboflavin, or pyridoxin, nor by the combined addition of all of these vitamins.

Bauernfeind & Norris (198) showed that the chick antidermatitis vitamin was essential for hatchability as well as for growth. Apparently little of the factor was needed for hatchability since the diet had to be heated at 120° (201) to destroy the chick antidermatitis vitamin before it became a limiting factor in hatchability.

Carter & O'Brien (217) have added the pigeon to the list of animals requiring the chick antidermatitis vitamin and they indicate that it is identical with vitamin B₃.

The observation by Jukes (202) that cereals contain the chick antidermatitis vitamin but that young shoots such as of barley do not, indicates that the vitamin is used up during germination, and is in line with the observation of Pratt & Williams (218) that pantothenic acid stimulates respiration of plant tissues (apple and potato).

Distribution.—Waisman and associates (219) studied the distribution of the chick antidermatitis vitamin in meat products and expressed their results in minimum amounts of material which protect chicks against dermatitis. Liver and kidney were found to be especially rich in the vitamin. About one-third of the potency of beef kidney was lost by stewing.

Chick *et al.* (236) showed that in pigs deficient in the filtrate factor, the hind quarters became paralyzed, indicating nerve degeneration in the pig also.

Peterson & Elvehjem (221) studied the effect of the medium on

the chick antidermatitis content of yeast. Yeast grown in a grain wort protected chicks against dermatitis at the level of 2 per cent; yeast grown in molasses protected at 4 per cent and yeast grown in a glucose-salt medium protected at 6 per cent. Commercial brewers' or bakers' yeast was effective at a 2 per cent level.

Lunde (220) and Kringstad & Lunde (199) have determined the distribution of the "rat growth factor" in fish products. Fish liver meal and cod roe were found to be good sources of the "rat growth factor." Sardines, kippers, and milt were fair sources.

Microorganisms.—Subbarow & Rane (222) showed that pantothenic acid was necessary for the growth of the Dochez NY5 strain of hemolytic streptococcus. Evans and associates (223) showed that certain exacting strains of *C. diphtheriae gravis* required pantothenic acid. McIlwain (225) has shown that pantothenic acid in high dilution promoted the growth of the "Richards" strain of *Streptococcus hemolyticus*.

Woolley (224) made the interesting observation that hemolytic streptococcus strain H69D could get along on only one half of the pantothenic acid molecule, the acid half. He suggested that this organism could be used as a test for the unknown acid part of the pantothenic acid molecule. By use of this test the acid, which is much more stable than the whole pantothenic acid molecule, can be fractionated with the object of isolation and determination of its structure; this would enable the synthesis of pantothenic acid.

McElroy & Goss (226) observed that in the sheep's rumen, the microorganisms were very active in the synthesis of the chick antidermatitis vitamin.

Is the "rat growth factor" multiple in nature? Does it differ from the chick and anti-grey hair factors?—To convincingly demonstrate that the "rat growth factor" is multiple in nature is no easy task. Such a demonstration would be convincing if the following conditions were met: (a) the vitamin must be fractionated into at least two fractions, each of which by itself must exhibit little activity; (b) the two "inactive" fractions when combined must exhibit activity.

It is obviously difficult to recognize that an "inactive" fraction does actually carry an active vitamin; yet it was precisely in this way that vitamin B was proved to be multiple in nature (227, 228). In this manner also vitamin G was shown to be multiple in nature (229), as well as vitamin B₆ (195).

So far only one basic test diet has been used to test for the "rat

growth factor," namely the usual synthetic diet with addition of all the members of the vitamin-B complex necessary for the rat except the "rat growth factor." Because of their availability, crystalline thiamin and riboflavin have been universally used. Such will also undoubtedly be the case with pyridoxin (vitamin B₆) now that it has been synthesized. Until now a concentrate or some natural food rich in pyridoxin, but low in the "rat growth factor," has been employed. With such a basal diet, rats should not grow at all or grow but slowly. Addition of concentrates containing the "rat growth factor" should stimulate the growth of such rats.

Hitchings & Subbarow (200) found that a crude preparation of the "rat growth factor" was more stable to acid at 100° than a more highly refined preparation. They tentatively proposed that the "rat growth factor" consisted of a stable factor and of one or more unstable factors. Their data lend themselves equally well to the interpretation that there is but one factor whose stability to acid varies with its purity.

Macrae *et al.* (194) found that the "rat growth factor" could be only partially extracted with amyl alcohol from an acidified liver extract. They tentatively assumed that there were two factors, one soluble in amyl alcohol and one insoluble. Since the same basic test was used to recognize the activity of both the soluble and insoluble fractions, the data may also be interpreted to mean that the same factor was present in the amyl alcohol extract as that which remained behind, perhaps as a complex insoluble in amyl alcohol.

Mohammad *et al.* (213) heated a relatively crude concentrate of the "rat growth factor" in 1 N NaOH and found the activity was not destroyed. They claimed that the "rat growth factor" must be different from the chick antidermatitis vitamin because under similar conditions (quoted Woolley *et al.*, 204) the chick antidermatitis vitamin was destroyed. They did not take into consideration that Woolley *et al.* (204) used a more highly refined preparation. Moreover, Macrae and associates (194) report the "rat growth factor" in their concentrates was readily destroyed by 5 per cent sodium hydroxide.

Kringstad & Lunde (199) could not extract the "rat growth factor" with ether from an acidified yeast concentrate. They concluded that it must be different from the chick antidermatitis vitamin since (quoting Woolley *et al.*, 230) this factor could be so extracted. However, both Hitchings & Subbarow (200) and Macrae *et al.* (194) have succeeded in extracting the "rat growth factor" with ether.

Lunde & Kringstad (211) suggested that the "rat growth factor" was more stable to heat than the anti-grey hair factor since an extract prepared by boiling brewers' yeast three hours produced as good growth over a seven-week period as the untreated yeast but allowed the rats to become grey, whereas the untreated yeast maintained the black color of the rats' fur. Inspection of their growth curves (211) showed considerable loss of the "rat growth factor" as measured by growth of the yeast extract as compared with the untreated yeast. The loss of "rat growth factor" in the yeast extract was compensated by raising the quantity of the extract administered. In the meantime, the rats were receiving inadequate "rat growth factor" which could well account for the rats' fur turning grey. The experiments were not continued sufficiently long to obtain the full effect of the increase in quantity of administered extract.

Oleson and associates (212) have studied the effect of various concentrates on greying and claim that the factor preventing greying (nutritional achromotrichia) is different from the other known members of the vitamin-B complex. They did not, however, indicate that they satisfied the rats' requirements for the "rat growth factor" with any of their concentrates.

An important communication by Mohammad and associates (213) shows that an ether extract of molasses promotes the same growth as the residue but the rats do not turn grey on the extract but do turn grey on the residue. Here it seems that a separation of the anti-grey hair factor from the "rat growth factor" has been made, the anti-grey hair factor being more soluble in ether. In considering the phenomenon of the greying of black rats' fur it is essential to remember:

(a) A syndrome is not necessarily associated with growth. It is well known, for example, that various degrees of thiamin insufficiency will cause varying amounts of growth, but if these levels are inadequate the rats will succumb with beriberi even after they have made considerable growth. Moreover, Stokstad & Manning (231) have shown with chicks that curly toe paralysis occurs most regularly when some riboflavin is in the ration, enabling the chicks to make considerable growth, even though the syndrome is curable by riboflavin. There is no reason to believe that the greying of rats is fundamentally different from other syndromes.

(b) Greying of rats' fur is influenced by other constituents of the diet, especially pyridoxin and nicotinic acid (211, 233).

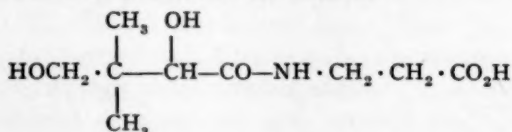
(c) Long experimental periods are required to get reliable results.

From available evidence it is more likely that the chick antidermatitis vitamin, the "rat growth factor," and pantothenic acid are all similar chemically rather than different. At any rate they all have the following properties in common: (a) solubility in ether from acid solution ["rat growth factor" (194), chick antidermatitis vitamin (230), and pantothenic acid (210)]; (b) adsorption on charcoal and elution with a fat solvent ["rat growth factor" (200), chick antidermatitis vitamin (230), and pantothenic acid (210)]; (c) lability to hot acid and alkali ["rat growth factor" (194), chick antidermatitis vitamin (204), pantothenic acid (206, 209)]; (d) acetylation and successful saponification ["rat growth factor" (194), chick antidermatitis vitamin (230), and pantothenic acid (210)]; (e) chloroform-soluble brucine derivative ["rat growth factor" (200), chick antidermatitis vitamin (208), and pantothenic acid (209)]; (f) solubility of metallic salts in various solvents ["rat growth factor" (194), chick antidermatitis vitamin (208), and pantothenic acid (209)].

This impressive list of common properties for the three factors must be considered as evidence which emphasizes the similarity of properties of the three factors. They do not prove identity. Only the preparation of one of the factors in pure form will answer the question.²

² As this manuscript was about to go to press, two papers appeared in *Science* of such great importance to the subject matter under consideration here that, at the Editor's suggestion, they have been included in this review.

R. J. Williams & R. T. Major, *Science*, **91**, 246 (1940), and D. W. Woolley, *Science*, **91**, 245 (1940), simultaneously announced that they succeeded in crystallizing the acid cleavage product of pantothenic acid. This they coupled with β -alanine to form physiologically active pantothenic acid. Williams & Major also announced the structure of the acid cleavage product and its synthesis. It proved to be α -hydroxy- β , β -dimethyl- γ -butyrolactone. When coupled with β -alanine, the structural formula of pantothenic acid would be:



The empirical formula of pantothenic acid would, therefore, be $\text{C}_9\text{H}_{17}\text{O}_5\text{N}$ instead of $\text{C}_8\text{H}_{14}\text{O}_5\text{N}$ (205) as previously suggested.

Whether pantothenic acid is identical with the anti-grey hair factor, the chick antidermatitis vitamin, and the "rat growth factor" or is but an active fraction of such concentrates will now be quickly determined as soon as synthetic pantothenic acid is made available.

VITAMIN C

Methods.—New methods for determining vitamin C were developed by Schulek & Floderer (237) who reduced ferric to ferrous salts, after which they added α, α' -dipyridyl; by Roe & Hall (238) who prepared the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid, and converted it to furfural which could be determined colorimetrically by the aniline acetate method. Chinn & Farmer (241) described a method for estimating ascorbic acid in the feces. Other work on methods has been published by Goldsmith *et al.* (239), Fujita *et al.* (240), and Woessner *et al.* (242).

Biological synthesis.—Musulin *et al.* (245) isolated a lipid type of substance from alfalfa meal which caused an increased rate of synthesis and excretion of ascorbic acid by rats on a milk diet. The active substance proved to be a volatile fraction of the nonsaponifiable matter.

Longenecker *et al.* (244) fed terpene-like cyclic ketones and found them to be very effective in stimulating ascorbic acid synthesis in rats. Among the most active were *d*- and *l*-carvone, *dl*-piperitone, isophorone, α - and β -ionone, pulegone, thujone, camphor, and nerolidol. An average excretion of 16.5 mg. ascorbic acid per day resulted from the feeding of 100 mg. of *d*-carvone. Aliphatic compounds such as diisobutyl ketone, dipropyl ketone, and dimethyl-acetyl carbinol were moderately active. These investigators thought it unlikely that these compounds served as precursors of ascorbic acid, but rather that they stimulated the synthesis of ascorbic acid from intermediate substances.

Rudra reported that manganese was important for ascorbic acid synthesis in the rat (246) and in germinating seeds (247).

Physiology.—There have been important contributions on the relation of vitamin C to blood lipase (248), the eye (275, 276), diphtheria toxin (249 to 252), and metabolism (253, 254, 255).

Human requirements.—Hoygaard & Rasmussen (243) studied the vitamin-C content of common Eskimo foods and the probable vitamin-C intake of the Eskimo. The Angmagssalik Eskimo receives a daily average dose of about 40 mg. of vitamin C in his food. Since about 15 mg. of ascorbic acid per day is considered sufficient to protect against scurvy, this disease is unknown among the Angmagssalik Eskimos. They get about 50 per cent of their daily vitamin C from marine algae and the other 50 per cent from the organs of mammals,

birds, and fish. On an exclusive meat diet, the intake is about 25 mg. of ascorbic acid per day.

Excretion studies as related to human requirements were made on normal human beings (256, 257), on human beings with kidney damage (258), on lactating women (259), on prematurely born infants (260), and on an experimental subject who had been on a vitamin-C deficient diet for 160 days.

Ascorbic acid and disease.—The effect of ascorbic acid was studied in tuberculosis (261, 262), in electrically induced fevers (263), in fever produced by injection of bacterial protein (264), in fevers resulting from disease (265). The ascorbic acid requirements were studied in patients with peptic ulcer (265) and achlorhydria (266).

Holmes *et al.* (267, 268) observed subclinical scorbutic symptoms in patients suffering from chronic lead poisoning. Administration of 100 mg. daily of ascorbic acid resulted in marked improvement in vigor, cheerfulness, in the color of the skin, and in the blood picture. The patients lost their irritability and nervousness, no longer had tremors, and experienced better appetite. Several cases of leukopenia were cured. Vitamin C seemed to react with toxic lead ions to form a poorly ionized and much less toxic compound. In the process, vitamin C was removed from the tissues and therefore an increased intake of vitamin C was required. The high content of lead in the urine dropped to normal, the lead presumably being eliminated in the feces.

Vitamin-C therapy was studied in essential hematuria (269), in experimental poliomyelitis (270, 273), in surgical operations (271), and in rheumatoid spondylitis (272). Wildbolz (274) made a systematic survey on the role of vitamin C in a number of surgical and urological cases.

OTHER WATER-SOLUBLE VITAMINS

Vitamin P.—The evidence upon which the existence of vitamin P is based has been reviewed by Scarborough (277), who claimed that the conclusion as to the reality of such a vitamin cannot be maintained on the basis of published work. He did show, however, that evidence from experiments on human subjects established the existence of a factor that decreases capillary fragility.

The influence of ascorbic acid and of citrin (vitamin P) on the capillary resistance of guinea pigs was studied by Zacho (278). Citrin alone showed distinct powers of increasing capillary resistance, but the simultaneous presence of both citrin and ascorbic acid was neces-

sary for the maintenance of normal capillary resistance. Citrin also prevented intestinal hemorrhage in scorbutic guinea pigs. It seemed that the hemorrhagic diathesis occurring in scorbutic guinea pigs was largely due to the lack of citrin, the other scorbutic symptoms developing from lack of ascorbic acid.

The grass juice factor.—Simplified diets for guinea pigs suitable for the assay of the grass juice factor were published by Kohler *et al.* (279). These diets were also found suitable for what appeared to be a new factor which was necessary for the prevention of stomach ulcers in guinea pigs. In another communication, Kohler *et al.* (280) reported that the grass juice factor could be extracted from dehydrated grass by acid acetone, but not by water, ether, or petroleum ether. Cannon & Emerson (281) have also studied with guinea pigs a factor in grass which seems to be the same as the grass juice factor.

Choline.—Choline continues to attract the attention of workers using the vitamin-B complex in nutrition experiments. Heretofore it has been an impurity in the concentrates of the vitamin-B complex. With the introduction of crystalline and highly purified components of the vitamin-B complex, choline was progressively eliminated until it has been reduced to the point where under certain conditions it proved to be a limiting factor. Such a case was reported by du Vigneaud *et al.* (282), who found that with some concentrates of the vitamin-B complex homocystine could support growth in rats on a methionine-free ration, while with other preparations, it could not do so until choline was added.

Vitamin M.—Additional work on vitamin M has been reported by Langston *et al.* (283), who showed by direct test that the nutritional cytopenia in monkeys on vitamin-M deficient diets cannot be prevented by thiamin, riboflavin, or nicotinic acid. They also argued that pyridoxin (vitamin B₆) is plentifully supplied in their vitamin-M deficient ration and especially in the modified Goldberger blacktongue-producing diet upon which nutritional cytopenia also developed. Since the basic vitamin-M deficient diet contains about 20 per cent of wheat as the only source of the vitamin-B complex, these investigators have, therefore, ruled out every factor except factor 2 (filtrate factor). Neither the vitamin-M deficient diet nor the Goldberger blacktongue-producing diet can be considered to contain adequate amounts of factor 2 (filtrate factor) without experimental verification. Yeast and liver extract, both rich in vitamin M, are among our best sources of factor 2.

Monkeys on a vitamin-M deficient diet developed bacillary dysentery (284). Also, on similar diets monkeys developed mouth lesions (285).

Vitamins L₁ and L₂.—The requirements of mice for the vitamin-L complex have been studied by Nakahara *et al.* (286); their requirements were found to be less than those of rats. These investigators (287) pointed out difficulties that are likely to be encountered in vitamin-L research. Bakers' yeast contains L₂ only and can be used to produce L₁ deficiency, but brewers' yeast cannot be used at all in vitamin-L research, since it contains both L₁ and L₂. Details with respect to diets and supplements for vitamin-L research are given.

Vitamins B₃ and B₅.—Carter & O'Brien (288) showed that vitamin B₃ is probably identical with the chick antidermatitis vitamin and vitamin B₅ with vitamin B₆ (pyridoxin).

A new vitamin for the chicken.—Bauernfeind & Norris (289) have described a new factor necessary for growing chicks and for hatchability of eggs. The factor is destroyed by dry heat at 120°. It is soluble in water, adsorbed on fuller's earth and is thrown out of solution with alcohol.

Vitamin H (curative factor for egg white injury).—The distribution of vitamin H in foodstuffs was studied by György (290). Autolysis of yeast in the presence of toluene liberates vitamin H in water-soluble form. Chloroform reversibly inhibits this apparently fermentative reaction.

György, Kuhn & Lederer (291) concentrated vitamin H from liver and obtained fractions potent in rats in daily doses of 30 to 40 µg. By electrodialysis studies, Birch & György (292) found that vitamin H has an isoelectric point between pH 2 and 4 and is an ampholyte which exhibits acidic properties.

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THE BIOCHEMISTRY OF MALIGNANT TISSUE

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TUMOUR METABOLISM

OXIDATION IN TUMOUR

The respiratory quotient (R.Q.) of tumour tissue was found in the tumours studied by Dickens & Šimer (1, 2) to be below unity, while normal tissue with similarly high glycolysis (including brain) had an R.Q. of unity. This result, which was interpreted as indicating a defective carbohydrate oxidation in tumours, has been disputed by Elliott & Baker (3). Using the Dixon-Keilin apparatus, they reported values for the R.Q. of rat cerebral cortex of 0.78 to 0.93 (mean 0.86) while with Philadelphia No. 1 sarcoma and Walker No. 256 rat carcinoma they obtained values of above 0.95 in seven out of twenty-eight tumours, twenty-one having R.Q.'s of 0.72 to 0.95. Their experimental technique, however, was criticised by Dickens (4) on assumptions supported by statistical examination of their data and of a large series of R.Q. measurements on normal rat brain cortex, all of which were close to unity.

Jares (5) has used a modified Fenn microrespirometer to determine the R.Q. of five types of experimental tumour. Among eighteen observations were included three with R.Q.'s of 0.99, 0.97, 0.95, respectively; the remaining fifteen had R.Q.'s between 0.91 and 0.75. Dickens & Šimer (1) also observed an R.Q. of 0.97 for a slow growing strain of Jensen sarcoma in phosphate medium—though lower values were obtained in bicarbonate (2)—which agrees with the average value of 0.95 obtained by Jares for a similar tumour.

For the R.Q. of normal lymph nodes of mice and of two lines of leukaemic lymph nodes Victor & Potter (6) report mean R.Q. values of 0.876 (normal), 0.840 (leukaemic line I), and 0.81 (leukaemic line M). It may be noted (7) that the mean anaerobic glycolysis of these strains was, for $Q_{G^{N_2}}$ values: 5.78 (normal), 12.76 (line I), and 19.78 (line M). The loss of ability to oxidise added glucose was complete in line-M leukaemia, but in the other strain it was not evident. These results are of interest in indicating that the effect of

the host on tumour metabolism may vary widely from one strain to another.

The conclusions of Elliott, Benoy & Baker (8) support the original view of Dickens & Šimer (1, 2) that there is a defective oxidation of carbohydrate, or of some carbohydrate intermediate, in tumour tissue. These authors found that oxidation of succinate to fumarate and of lactate to pyruvate appeared to be defective. On the other hand they produced evidence that succinate formation from pyruvate occurred and that the fumarate-malate equilibrium was unimpaired. Synthesis of carbohydrate from lactate or pyruvate did not occur in isolated brain, testis, or rat tumour tissue (9).

Euler, Adler & Günther (10), however, considered that the *apo*-dehydrogenase systems of Jensen sarcoma towards lactic and malic acids showed no essential difference from those of muscle. Nevertheless according to Euler, Malmberg, Günther & Nystrom (11) the hydrogen donators (lactic acid, etc.), are always in excess in Jensen sarcoma tissue while the amounts of cozymase and flavin enzyme are insufficient for the full activation; cozymase is thus present mainly as dihydrocozymase (ratio from 1/4 to 1/10) whereas in muscle the ratio of oxidised to reduced cozymase is below unity.

In the presence of cozymase and flavin, a dialysed extract of Jensen sarcoma rapidly oxidised malic and lactic acids, but not succinate, glucose, or fructose. When coenzyme II was added together with flavin enzyme, hexosemonophosphate and hexosediphosphate were rapidly oxidised. Glycogen was also oxidised, presumably with the intermediate formation of Robison ester. Although Jensen sarcoma contains large amounts (see below) of coenzymes I and II, these are rapidly inactivated after excision or destruction of the tissue (cf. 12); it is suggested (13) that this is the reason that the strongly active hexosemonophosphate dehydrogenase system in tumour has hitherto been overlooked. In ground-up brain tissue the system appears to be relatively stable (14).

Euler & Bauer (15), using the Thunberg method with methylene blue, found that whether or not phosphate was added, the rate of decolourisation was much greater with Jensen sarcoma extract and glycogen than it was with glucose as substrate, though Robison ester is a much more powerful hydrogen donator than either. Magnesium plus manganese and adenylic acid accelerated the reaction with glycogen as substrate. Phosphorylation of glycogen seems to occur even in the absence of added coenzyme, and the formation of hexosemono-

phosphate is probably an intermediate stage in this oxidation, following primary formation of the Cori ester.

Further comparative experiments (16) on the phosphorylation of glycogen support this view: in muscle extract it is much faster than in sarcoma extract; and in sarcoma there is a tendency for the relative amount of easily hydrolysable phosphorus (Cori ester) in relation to the total ester phosphorus to increase, suggesting that the transformation of the 1- to the 6-glucose phosphate may be delayed or inhibited in the sarcoma extract.

Succinic dehydrogenase in tumour tissue is very variable, but on the whole its activity is less than that of normal tissue (10). Similar results have been obtained with the complete oxidase system (17, 18).

In spite of previous conflicting evidence, the content of cytochrome-*c* is reported (19) to be low in viscera and in tumours (6.78 to below 0.15 mg. per cent), and also in carcinoma and fetal tissues (20). The oxidation, of course, is governed not solely by this component but by the whole oxygen activating system, which has been partially analysed in Jensen sarcoma extract (16, 21). Diaphorase is removed from sarcoma extract by chromatographic adsorption on calcium carbonate, and thus an extract is obtained which contains succinic dehydrogenase and diaphorase in the ratio of ten to one. In this system addition of cytochrome-*c* is not sufficient to bring about activation; cytochrome-*a* and -*b* and cytochrome oxidase are also necessary for succinate oxidation. The lack of complete activation in the tumour is due to the absence of this complete cytochrome system. Glutathione is not catalytically active, but diaphorase is an essential part of the system and in the presence of the other components it brings about fully activated oxygen uptake.

The lactic acid system also requires the above components. When diaphorase and cytochrome-*a*, -*b*, and -*c* are present together with cytochrome oxidase, the oxygen uptake by Jensen sarcoma extract with *l*(+)-lactate as substrate is almost the same as when muscle extract replaces the sarcoma extract. With *d*(-)-lactate little or no oxygen uptake occurs.

Experiments (22) on the addition of C_4 dicarboxylic acids to minced or sliced tumour tissues showed that an increase of respiration was caused by fumarate or malate, and a decrease by malonate. It was considered that the loss of oxygen-transporting C_4 acids by diffusion was compensated for by their addition.

Elliott and co-workers (23, 24, 25, 26, 27) have studied various

tissues, including tumours, to see whether succinate, fumarate, and oxalacetate are on the path of lactate and pyruvate oxidation, as in the Toennissen-Brinkman cycle. Tumour, in common with a number of normal tissues, did not metabolise these substrates in the same cycle of reactions as that found in the kidney. Although the results do not prove that the cycles proposed by Szent-Györgyi or Krebs play no part in tumour metabolism, it is unlikely that they are the main route of oxidation, at any rate in certain tumours (27).

Breusch (17, 28) found that a mouse tumour was unable to reduce oxalacetate or oxidise fumarate, and that this was true also of normal lung tissue. Tumour had only 20 per cent of the ability of muscle to dehydrogenate succinate. α -Ketoglutaric acid and citric acid did not increase the oxygen consumption and were not degraded, nor was the reduction of oxalacetate brought about by the addition of coenzyme, though respiration was thereby increased by 40 per cent. Greville (29) has shown that even in the presence of glucose, which provides carbohydrate breakdown products as hydrogen donors, the removal of oxalacetate is not accelerated in Jensen sarcoma.

Finally, Szent-Györgyi (30) has suggested that tumours may utilise a metabolite, e.g., lactate, which will reduce fumarate but not the more negative oxalacetate system. It is at any rate clear that if the four-carbon system operates in tumour it has not the same intensity as in muscle.

Ciaranfi (31) has found that the respiration of tumour slices is not increased by the addition of monocarboxylic saturated or unsaturated fatty acids to the medium, and thinks that lipids in tumours are of importance for cell structure but not for metabolism.

MECHANISM OF TUMOUR GLYCOLYSIS

Knowledge of the intermediate processes in tumour glycolysis, though still less complete than for muscle, has been greatly extended by the preparation of extracts which possess glycolytic activity similar to that of the tumours from which they were made (32). This was made possible by the addition of considerable quantities of adenylic acid and cozymase, which in aqueous tumour extracts are subject to rapid destruction by enzymes which attack adenine compounds and nucleotides (12). Since all tumours examined were found to contain zymohexase, which rapidly converts hexosediphosphate into triosephosphate (33), and since fresh tumour is known to contain cozymase (see below) there appears to be little reason to doubt that a phos-

phorylating glycolysis similar in principal to that in muscle may also occur in tumour tissue. The rapid abolition of tumor glycolysis, which occurs on destruction of the tissue by freezing, grinding, etc., is thus apparently due to inactivation of these coenzymes, the stability of which is dependent in some way upon the integrity of cell structure. Euler & Schlenk (13) have shown a similar rapid destruction of coenzymes I and II in tumour extracts and also attribute to this the fact that phosphorylating metabolism in tumour (as in brain and embryo) has hitherto been relegated to a secondary position (34, 35, 36).

Nonphosphorylating glucolysis is considered by these authors to be the principal route of lactic acid fermentation in embryo, brain, and tumour tissue. In embryo tissue Needham & Lehmann (35) were unable to detect cozymase, but their evidence for this has been criticised by Boyland (37); it is certainly present in tumour tissue. Glyceraldehyde was found to inhibit tumour glycolysis (38) in 0.001 *M* concentration while twenty times this concentration was without effect on respiration of normal and malignant cells. This inhibition could be reversed by small amounts of pyruvate (39). Ashford (40) showed inhibition of glucose fermentation in brain tissue.

On the basis of these findings and a detailed consideration of phosphate balance Needham & Lehmann (35) regarded glyceraldehyde as a specific inhibitor for the nonphosphorylating type of glycolysis, distinguishing it from that type of glucose or glycogen metabolism in which hexosephosphate is the intermediate; they considered that the glycolysis of embryo, like that of tumour, was inhibited whereas that of yeast and muscle was not. Baker (41) also found an inhibition of respiration and glycolysis in tumour, brain, testis, and embryo by glyceraldehyde. It has been shown that yeast and muscle fermentation can also be inhibited by glyceraldehyde (42, 43, 44), and there is other evidence (42) that phosphorylation must occur in brain glycolysis. Macfarlane (45) has clearly indicated the need for careful consideration of the difference between phosphorylation in the living cell and in tissue extracts. She gives excellent reasons for supposing that in many intact cells phosphorylation may proceed with the participation of only exceedingly small amounts of free phosphate; the argument cannot well be summarized but merits careful study. This paper also contains a reply to the criticism of Lehmann & Needham (46) that the concentrations of glyceraldehyde required for inhibition of the fermentation in yeast and muscle is so high as to lead to a nonspecific inhibition.

It is of interest that only the *l*-component of glyceraldehyde is effective in inhibiting glycolysis (46, 47, 48). The explanation of this is uncertain as yet (cf. 45).

Holmes (49, 50) has found that different tumour strains show great differences in their relative rates of attack on hexosephosphates as compared with glucose or glycogen, and tends to favour Needham's view that different mechanisms are concerned, though her results are inconclusive. Hitchings, Oster & Salter (51) found that saline tumour extracts failed to catalyse the reaction between triose-phosphate and pyruvic acid: their extracts, unlike Boyland's aqueous ones, did not contain coenzyme-destroying enzymes, and they concluded that tumour and muscle glycolysis follow different metabolic pathways.

Mawson (52, 53) on the basis of glutathione catalysis of lactic acid formation by tumour extracts at 52° C., thinks that the glyoxalase system is on the main pathway of lactic fermentation in tumour. This interpretation has been criticised (37, 48, 51).

The question of phosphorylating or nonphosphorylating glycolysis in tumour tissue must therefore be regarded as still open, though the balance of evidence is now with the former school, in view of their successful preparation of the phosphorylating type of glycolytic extract from tumours. Whether the exact intermediate course is the same in muscle and tumour remains controversial. Quite recently Meyerhof & Perdigon (53a) have obtained extracts from rat and chick embryo which transform hexosediphosphate and pyruvic acid into phosphoglyceric acid and lactic acid in the presence of sodium fluoride, at a rate equal to or greater than that of the embryonic tissue itself. Thus even in embryonic tissues a phosphorylating glycolysis appears probable.

With intact tumour slices, only glucose, mannose, and to a variable extent, fructose, are converted into lactic acid (43, 54). It is highly probable that the relative lack of attack on hexosemonophosphates, hexosediphosphates, starch, and glycogen (55) which are added to the surrounding fluid is due to their nonpenetration of the cells. Even in the tissue-slice method some cell damage is unavoidable, and with progressive destruction of the tissue one would expect to find an increasing accessibility of the enzymes to their substrates, offset to a variable degree by loss of coenzyme activity and of carriers, caused by destruction and dilution. Experiments of Boyland & Boyland (43), in which lactic acid formation by tumour slices and by extracts

were compared, are illuminating in this respect. Active glycolytic extracts of tumour, unlike the whole tissue, produce lactic acid at about the same rate from glucose, mannose, glycogen, hexosemonophosphate, and hexosediphosphate. These authors attribute the action on glycogen to the amylase activity of cancer tissue (56), while Euler & Bauer (15) appear to favour a phosphorolysis of glycogen similar to that in muscle.

The fermentation of fructose by Crocker sarcoma tissue (43) appears also to be limited by the slight permeability to fructose as compared with permeability to glucose. In Jensen sarcoma, enzymatic differences probably also restrict fructose fermentation (54); and since oxidation of concentrations of fructose which are not appreciably glycolysed anaerobically occurs in brain, retina, and testis (57), it may be inferred that anaerobic conditions will also tend to diminish the permeability of tumour tissue to fructose.

Aerobic glycolysis.—It becomes increasingly clear that aerobic glycolysis can no longer be considered as a specific property of cancer tissue (cf. 58) although practically all tumours glycolyse aerobically (59) and relatively few normal tissues do so. Muscle and nerve may reasonably be excluded from the latter category and also salivary glands (60, 61), for in these tissues the active metabolism is distinct from that at rest. Aerobic glycolysis may arise from physical or chemical injury to tissues, and that of retina, testis, and embryo has been explained on these grounds; but the recent demonstrations that medulla of kidney has a strong aerobic glycolysis, not only in Ringer solution (62) but also in the serum of the same species (63), and that cartilage and intestinal mucosa (66) also glycolyse aerobically, show clearly that the development of aerobic glycolysis may be in such cases the normal physiological response to an oxidative metabolism which is inadequate to meet the energy requirements of the tissue.

Such tissues have a metabolism which would formerly (59) have been considered typical of benign tumours. To the same category appear to belong normal and papillomatous skin (67, 68), which undergo little striking change of metabolism on transformation from normal tissue to a benign tumour, but which nevertheless appear to suffer a definite metabolic change when transformed into a malignant tumour (cf. 67). By the use of an ultramicromanometric method, Berenblum & Chain have been able to study the metabolism of the isolated epithelium; details are not yet available.

Nakatani *et al.* (69) have studied the metabolism of liver tissue

undergoing malignant change induced by feeding dimethylaminoazobenzene. Although the respiration remained unchanged, the aerobic and anaerobic glycolysis increased when hepatomas appeared. This has been confirmed by Orr & Stickland (70) who, however, have left it undecided whether the changes reported by the Japanese workers in metabolism of the intermediate cirrhotic stage are significant.

Aerobic glycolysis of tumours is depressed by pyocyanine (N-methyl α -oxyphenazine) (71, 72) and by simple N-alkyl phenazonium salts (73, 74), with some increase of respiration and also with variable extents of depression of the anaerobic glycolysis. It is perhaps significant that these semiquinone-forming systems can act as substitutes for the flavin enzyme in the hexosemonophosphate system (74), although other dyes of similar potential are very ineffective carriers. Inefficient oxidation of Robison ester caused by lack of suitable oxygen-activating systems may thus have been artificially overcome in the tissue by the addition of phenazine methochloride; investigations on this are in progress.

Another group of phenazine compounds (and also quinoline, acridine, and pyridine compounds) containing basic substituents, e.g., phenosafranine, has the opposite result (in $0.00001 M$ solutions), for these substances increase aerobic glycolysis, even in tumour tissues, and thus abolish the Pasteur effect (73). Among these is the carcinogenic dye, styryl 430, the action of which, however, is limited to the cerebral cortex, as is also that of cations, especially potassium (75), disturbance of salt balance (76), ammonium ions (66), and guanidinium ions (77).

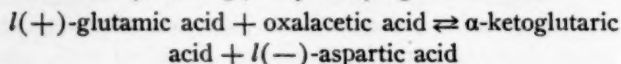
The action of various other oxidation-reduction systems on the metabolism of tumour tissue has also been studied (5, 72, 78). Ferricyanide ($0.01 M$) inhibits specifically the aerobic glycolysis of tumour cells without affecting their anaerobic glycolysis (79, 80). The inhibition is, however, not complete (72, 81). Maleic acid inhibits the respiration slightly and increases aerobic glycolysis in tumours and brain slices (66).

Sulphydryl compounds (82) increase areobic fermentation in yeast cells. Similarly a striking effect has been reported for tumour tissue in the presence of reduced glutathione (83). Other workers have failed to confirm these results (66, 84).

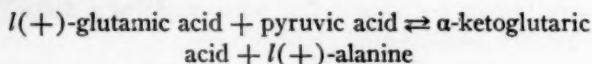
Transamination.—This important metabolic exchange of amino groups between an amino acid, e.g., glutamic or aspartic, and a keto acid, resulting in ketoglutaric or oxalacetic acid, respectively, and in

the reductive amination of the keto acid, was first described by Braunshtein & Kritsman (85, 86). The transaminating enzyme occurred in all tissues studied, except nucleated erythrocytes and malignant tumours.

The Jensen sarcoma also caused some transamination though it was less active than muscle (87). Euler and associates repeated these investigations with the nonnatural *d*(—) as well as with the *l*(+)-glutamic acid (16, 88, 89) since the partially racemised glutamic acid occurs in tumour (*v.i.*, Kögl). By studying the reaction



they found, on the basis of semiquantitative measurement of the disappearance of added oxalacetate, that the *l*(+) acid was much more rapidly transaminated in Jensen sarcoma extract than *d*(—)-glutamic acid. The difference in rate was, however, less pronounced with sarcoma than with muscle extract. Transamination of *l*(—)-aspartic acid with pyruvic or ketoglutaric acid also occurred in sarcoma as in muscle, but the reaction



occurred only in muscle, not in Jensen sarcoma or Brown-Pearce rabbit tumour extracts (16).

Similarly, glutamic dehydrogenase in the tumours was shown to be specific for the *l*(+) acid. Dickens & Weil-Malherbe (90), however, had found that both optical forms were slowly dehydrogenated in Jensen sarcoma extract; in their experiments it is now evident that there must have been lack of coenzyme.

Braunshtein (91) pointed out that quantitative experiments are necessary to decide points raised by Euler *et al.* More extensive results on minced tumour tissue (92) confirmed the previous report (86) that tumour tissue in some instances completely failed to effect transamination. In all other cases the rate of amino-group transfer was markedly inferior to that of normal tissues; usually less than 15 per cent of the amino nitrogen was transferred during a two-hour period. But the extent of transamination of the *l*-amino acids, only, was lowered, while that of the *d*-isomerides appeared on the whole to occur at about the same or slightly higher rate than in normal muscle. [Cohen (93) denies that all *l*- α -amino acids or that any *d*-amino acids are active in transamination in muscle.] On the average these results

for malignant tissue show a relative shift of the reaction rate in favour of the *d*-isomerides (mean ratio $l/d = 1.11$; for muscle it is about 7 to 8). Although this result would appear to show some conformity with the presence of partially racemised protein in tumour tissue (Kögl), the weakness of the reaction in tumours and its slight effect on optical activity make it unlikely that this would account for the stereochemical abnormality claimed for tumour proteins.

TUMOUR CONSTITUENTS

The optical form of amino acids in tumours.—Striking and important claims have been made by Kögl & Erxleben (94) and Kögl (95) who have reported the isolation of partially racemised amino acids, particularly glutamic acid, from tumour protein, whereas normal and embryonic tissues gave the "natural" *l*-acids. They found that 10 per cent or more of the total amino acids obtained after a short acid hydrolysis of the protein from tumours of man, rabbit, and mouse was in the racemic form. The *d*-forms of leucine, lysine, valine, hydroxyglutamic acid, and arginine were present in amounts up to 5 per cent, while *d*-glutamic acid accounted for a large fraction (up to 45 per cent) of the total glutamic acid.

Proteins of healthy tissue yielded no racemic amino acids other than the traces formed during the acid hydrolysis, and from these tissues relatively good yields were obtained of pure *l*(+)-glutamic acid ($[\alpha]_D + 31.6^\circ$ in hydrochloric acid solution). On the other hand an ovarian tumour yielded glutamic acid of optical activity $[\alpha]_D + 21.8^\circ$, an ovarian carcinoma gave glutamic acid of optical activity $[\alpha]_D + 4.6^\circ$, and two specimens of mammary carcinoma yielded glutamic acid of optical activity $[\alpha]_D + 11.6^\circ$ and $[\alpha]_D + 16.1^\circ$. Later experiments by Kögl & Erxleben (96, 97) showed that after destruction, by yeast fermentation, of the *l* component of partially racemised glutamic acid which had been isolated from Brown-Pearce rabbit tumours, pure *d*(-)-glutamic acid ($[\alpha]_D - 31.2^\circ$) was left.

The great significance of these results is evident, if it is correct that the tumour protein is abnormal in being built up partially of the "nonnatural" optical isomerides of the amino acids. Kögl & Erxleben suggested that cancer cells show a fundamental difference from normal healthy cells in that cancer cells do not discriminate between the incorporation of natural and nonnatural amino acids. They advanced theories which are based on this peculiarity to account for the autonomous growth and invasion of malignant tumours.

These findings have not yet been generally accepted because workers in England (98) and in the United States (99) have independently obtained the "natural" optically pure glutamic acid from ten specimens of tumour. Kögl & Erxleben (96, 97), in reply to these criticisms, pointed out that there was an essential difference from their original method in the technique that was used to isolate glutamic acid in both the above sets of experiments. Kögl & Erxleben originally used a butyl alcohol extraction of the protein hydrolysate, from which the glutamic acid was crystallised as hydrochloride. On the other hand, the above-mentioned critics used Foreman's method for isolating monoaminodicarboxylic acids by precipitation of the calcium or barium salt from aqueous alcohol. Even when Kögl & Erxleben (96, 97) applied this technique to the hydrolysate of a tissue to which a considerable proportion of *dl*-glutamic acid had been added, they obtained optically pure *l*-glutamic acid. This result they attribute to the greater solubility of calcium *d*-glutamate in dilute alcohol and to the poor crystallising power of *dl*-glutamic acid (as compared with *l*-glutamic acid) from the hydrochloric acid solution.

Confirmation of the isolation of partially racemised glutamic acid from tumour hydrolysates has been obtained by White & White (100). Using Kögl & Erxleben's original technique they found amounts of 34.6 and 15.8 per cent *d*-glutamic acid in hydrolysates of mouse and human mammary carcinomata, whereas by the barium salt method they isolated only the pure *l*-glutamic acid. Thus it appears that the findings of Kögl & Erxleben have been confirmed.

Chibnall and his collaborators (personal communication) then decided to make a further detailed research into this problem. In the first instance they investigated the solubility of the calcium salt of both racemic and natural glutamic acids in aqueous alcohol, and contrary to the findings of the Dutch workers they found that the racemic salt was completely precipitated and that it behaved in exactly the same way as the salt of the natural acid. Turning to the solubility of glutamic acid in hydrochloric acid, they found that the racemic hydrochloride was slightly more soluble than the natural hydrochloride. They were therefore willing to concede that this fact might explain some of the discrepancies, but they considered that the amount left in the mother liquors would not be sufficient to account for the difference in the results obtained by the two groups of workers. They decided to make a quantitative separation of the glutamic acid from the protein material from metastases obtained

from the liver of a woman dying from carcinoma of the colon. This complicated and laborious task revealed the presence of a small amount of unnatural acid, which accounted for only 0.3 per cent of the total glutamic acid present. The fact remains, however, that they confirmed the presence of minute amounts of racemic acid in the hydrolysis products.

The analysis and separation was repeated by the method of Dakin, and again a small yield of the unnatural acid was obtained. It would appear from these results that they had succeeded in confirming the claim of the Dutch workers that unnatural acid is present in tumour tissue proteins.

Chibnall and his collaborators then turned their attention to the glutamic acid of proteins obtained from nonmalignant tissue. They found that tissue obtained from ox heart muscle, rabbit myosin, and seed proteins all showed the presence of small quantities of racemised glutamic acid. They therefore concluded that while the Dutch workers had definitely shown the presence of racemic glutamic acid in malignant tissue, this could not be regarded as a specific difference between normal and malignant tissue.

At the present time the subject is under intensive investigation in the laboratories of the two groups of workers, and the final conclusions cannot yet be given. It would appear, however, that the specificity of the observation has been seriously shaken by the work of Chibnall and his collaborators.

Amino acids and nucleic acid.—The nucleic acid of malignant tissue is identical with thymus-nucleic acid (101). The extractable arginine of Jensen sarcoma is 0.0385 mg. per gm. fresh weight while that of rat liver is from 0.0 to 0.0047 mg. per gm.; the higher arginine content of the sarcoma extract is attributed to the presence of a histone containing 14 per cent arginine (102). Equal amounts of tryptophane are present in active and necrotic tumour areas (103), but more has been found in the protein of necrotic (1.16 per cent) than of nonnecrotic (0.827 per cent) Jensen sarcoma tissue (104). Lang (105) gives similar figures for human carcinoma; Jensen sarcoma had 0.81 per cent and various normal rat tissues contained from 1.21 to 0.86 per cent tryptophane.

Coenzymes.—The coenzyme-I contents of Jensen-sarcoma and Crocker-sarcoma extracts (ratio of tissue to extract is unity) are found to be 110 and 65 Co-units per ml. (32). Euler & Schlenk (13) found in Jensen sarcoma per gm. of tissue: 160 µg. coenzyme I (of

which 0.75 to 0.90 was in the reduced form), 80 μ g. coenzyme II, 0.6 to 2 μ g. aneurin, 2.2 μ g. cocarboxylase, and 2 μ g. flavin or flavin enzyme. The flavin content of rat liver tumours induced by feeding dimethylaminoazobenzene is lower than that of normal liver: the tumour contained no cocarboxylase, while the liver had 1.4 μ g. per gm. (106). The amount in the tumours studied by Euler & Schlenk was, however, similar to that in normal tissues.

Reducing substances in tumours.—The substance in tumour tissue which reduces phenol-2,6-dichlorophenolindophenol was originally considered (107) to be ascorbic acid. Feeding experiments suggested that a large part of this fraction was some constituent as yet unidentified (108). Desiccation of the Jensen sarcoma tissue unfortunately caused loss of half of the reducing power, but enabled the remaining reduction to be entirely accounted for as ascorbic acid by biological test (109). From spectroscopic and biological examination of aqueous extracts of Jensen sarcoma tissue it was concluded that the only indophenol-reducing substance was ascorbic acid (110). A similar agreement was found for Philadelphia No. 1 sarcoma (111). The low ascorbic assays of Harris were probably due to incomplete consumption and absorption of minced tissue by the guinea pig (37).

When tumour-bearing guinea pigs are kept on a scorbutic diet, the reducing power of the tumours falls with that of the tissues (109, 112) and is restored to normal level on injection of ascorbic acid.

The ratio of molar concentrations of ascorbic acid to glutathione in tumours and in brain is about unity (107, 113). In most other tissues except the adrenal there is a large excess of glutathione.

In the hepatoma produced by feeding dimethylaminoazobenzene to rats, the glutathione and ascorbic acid contents were greatest in the early stages (thirty to forty days) of the tumour (114). The hepatomas contained 0.40 mg. ascorbic acid and 2.43 mg. glutathione per gm. tissue whereas normal liver had only 0.18 and 1.71 mg. respectively (115). Excess dietary ascorbic acid increased the content of ascorbic acid in the tumour more than in healthy liver tissue, although the converse applied to cysteine feeding.

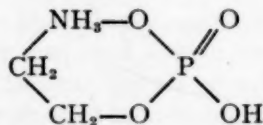
A single intraperitoneal injection into mice of 0.25 mg. of 3,4,5,6-dibenzcarbazole caused an increase of glutathione in the liver which was maximal during the succeeding twenty days and frequently led to bile-duct hypertrophy. Ascorbic acid and cholesterol contents of the liver were not affected; injection of other carcinogenic compounds did not increase the liver glutathione (116).

After injection of flavin and flavin enzyme into rat sarcoma the content of reductone (ascorbic acid + $C_3H_4O_3$) fell markedly (11).

Lipids.—The total cholesterol and neutral fat contents of Jensen sarcoma increase with the age of the transplant, while the phospholipid fraction diminishes (117). The necrotic centre of Jensen sarcoma has from two to two and a half times the cholesterol content of the actively growing margin, and growth *in vitro* of the sarcoma cells does not alter the cholesterol content of the culture medium (118). In spite of their high sterol content, mouse and rat implanted tumours are unable to store cholesterol added to the diet (118a, 118b). Ichiba & Somekawa (119) obtained an oily extract from fresh rat sarcoma tissue which contained cholesteryl palmitate, cholesterol, and a solid alcohol, besides liquid esters. Hydrolysis gave 44.3 per cent of liquid and 55.6 per cent of solid acids, including stearic acid and an acid of m.p. 40° . They stated that 50 per cent of the cholesterol occurred as ester.

Sueyosi & Miura (120) have applied their method of fractionation of the bromides of unsaturated fatty acids to the phosphatides derived from rabbit sarcoma, and have obtained mainly the dibromide accompanied only by a little tetrabromide. Human uterine myoma also yielded less tetrabromide and more dibromide than the corresponding uterine muscle.

Aminoethylalcohol phosphoric acid.—Outhouse (121, 122, 123) has isolated this interesting compound from malignant tissues. The compound gives an insoluble lead salt which is precipitated together with that of an aminohexahydric alcohol phosphoric acid, which may be a hexosamine compound. Aminoethylalcohol phosphoric acid is considered to exist between pH 5 and 9, as



It is identical with the synthetic product made from aminoethylalcohol and phosphorus oxychloride, m.p. 224° (cor.), and hydrolysis of the natural substance by phosphatase gives aminoethylalcohol.

The phosphoric ester could not be detected in pancreas, liver, placenta, or embryo, but malignant tissues contained, on an average, 36 mg. per 100 gm. of tissue. It appears, however, that the substance

is not, as was at first thought, specific for cancer tissue, for Colowick & Cori (123a) have recently shown by fractionation with uranium acetate that aminoethylphosphoric ester constitutes 25 per cent of the acid-soluble organic phosphate of rabbit small intestine. Nothing is known of its functions or source; for the latter, kephalin would be an obvious guess, though Pourbaix (124) conjectures a formation from phosphopyruvic acid.

Inorganic constituents.—Spectroscopic observations show that the copper content of tumours varies widely, from 0.5 to 20 $\mu\text{g.}$ per gm. of fresh weight (125, 126). The corresponding copper content of active Jensen-sarcoma tissue is 0.32 $\mu\text{g.}$, and of the necrotic areas 0.73 $\mu\text{g.}$ Rat liver contains from 1 to 6 $\mu\text{g.}$ per gm.

Lasnitzki (127) now finds that the proportions of the radioactive isotope of potassium to total potassium in Jensen sarcoma, rat muscle, and mineral potassium chloride are not appreciably different.

Microincineration studies of tumour and normal tissues have been reported by Hueper (128) and Cathie & Dawson (129).

Physical properties and diffusion factor.—Voegtlin and co-workers have determined the pH of tissues *in vivo* by means of the capillary glass electrode (130). In this way they have been able to study the influence of parenteral administrations of a number of sugars on the pH of malignant tissues (131). They have further studied the influence of pH on the reversal of proteolysis, and have found that proteolysis occurs in oxygenated extracts of normal and neoplastic tissues only within the physiological range of pH (132). Bierich & Lang (133) have tabulated E_h values for various rat and human tumour tissues and do not find any lower reduction intensity than in the normal cell. Shear (134) and Belkin & Shear (135) made the rather surprising observation that swelling of normal and tumour cells occurs in both hypertonic and hypotonic electrolyte solutions, but is retarded or inhibited by the presence of protein; the effect of the protein is reversible. On the basis of the effect of hypotonicity on phosphate diffusion measurements, Smoilovskaja (136) reported that tumour cells are less permeable than normal cells. The bioelectric potentials between various points on the skin of mice undergo a transient change of several millivolts in the region in which a mammary carcinoma or a methylcholanthrene-induced tumor is appearing (137, 138). The presence of the diffusion factor, which increases the permeability of the dermis, has been demonstrated in rapidly growing mammalian tumours in amounts greater than in any normal

tissue except testis, though it is absent from the rapidly growing fowl sarcoma No. 1 (139).

ACTIVATORS AND INHIBITORS OF METABOLISM

Certain substances belonging to this class have already been mentioned. Coenzyme-T preparations which are free from pyruvic acid have now been obtained from ox brain (140), an improved preparation of which 0.1 to 1 μ g. caused increases of 30 per cent in rat brain glycolysis (141). The various preparations were far from pure, however, for their nitrogen content varied from 3 to 12 per cent. The preparations contain or are associated with protein.

Berenblum, Kendall & Orr (142) have studied the action of a number of irritants with anticarcinogenic action (e.g., mustard gas, cantharidin) on minced-tumour metabolism. Although glycolysis was diminished by these substances more than by other irritants, ethylene bis- β -chloroethyl sulphide had less effect. The oxidative metabolism of tumour tissue which had been exposed to poisons (potassium cyanide, mustard gas, x-rays, and sodium fluoride) tended to approach values of normal tissues (143). Injection of colchicine into tumour-bearing animals caused depressed tumour respiration, though that of the liver tissue was unaffected (144). The effect of a number of inhibitors of tumour growth on respiration and glycolysis of mouse tumours was found to be small, even when the growth was strongly inhibited (145). The inhibitors used included 1,2,5,6-dibenzanthracene, 1,2,5,6-dibenzfluorene, sodium sulphanilylsulphanilate, α -nitroso- β -naphthol, and 4,4'-diamino-2,2'-dinitrophenylmethane. The inhibition of growth produced by Trypan blue was accompanied by a decrease in respiration and glycolysis, while that caused by *p,p'*-diaminodiphenyl sulphoxide was accompanied by diminished glycolysis. Healthy tissue, free from necrotic material, was selected for these measurements; no obvious difference in the extent of necrosis in normal and inhibited tumours was observed, but this point is being more closely investigated. The same authors found no great difference in the *in vitro* metabolism of various strains of grafted tumours which varied widely in their rates of growth.

X-radiation produces a definite decrease in the R.Q. of Philadelphia No. 1 sarcoma of the rat, together with an increase in aerobic glycolysis (146). Irradiation with ultraviolet light destroys preferentially the glutamic dehydrogenase which attacks the *l*(+) acid (147); this type of effect may be significant in the etiology of cancer (148).

Pourbaix (124) has discussed his results on the inhibition of yeast and tissue metabolism by carcinogenic agents. He found that the oxidation and fermentation of yeast were at first inhibited by the addition of styryl 430, but after several hours resumed their normal intensity. The fermentation which appeared, however, was of a different type—it was insensitive to sodium fluoride, probably because phosphorylating coenzymes had been inactivated or destroyed so that intermediate formation of hexosediphosphate no longer occurred. Dickens (73), however, observed accelerated respiration and glycolysis in brain tissue exposed to styryl 430.

Sodium selenite ($0.00001 M$) was found to inhibit tumour respiration (149). A similar concentration of dinitro-*o*-cresol greatly increased aerobic glycolysis and respiration of tumour tissue (150). It had been reported that dinitrophenol administered to tumour-bearing rats caused "destructive cellular changes whose significance remains undetermined" (151), but other observations indicated that there was no loss of tumour weight even when body weight fell (152) as a result of the use of general metabolic stimulants, including nitrophenols, and there was, in fact, a tendency for earlier tumour formation (153).

A large number of publications have described the so-called "intercellular hormones" (154) which are liberated when cells are subjected to various kinds of injury. Cell-free filtrates are thus obtained which appear to contain at least three factors which stimulate respectively cellular proliferation (155 to 159), respiration (160 to 163), and glycolysis (164).

Whether the proliferation-promoting substances are obtained from animal tissues or yeast they seem to be related to nucleic acid derivatives; the yeast-factor contains guanine, adenine, pentose, and phosphorus, and is free from pyrimidines, protein, sulphur, or pyridine (165, 166, 166a). There is some chemical resemblance to the active fraction of chicken tumour I [Claude (167)] and to the same author's fractions of chick and rat embryos. According to Ruddy (168) the respiratory stimulator, which is obtained from crude "bios" preparations, malt combings, and animal tissues, is specific, and is distinct from the above proliferation-promoting fractions. Further purification of all these fractions will no doubt reveal more clearly their relation to known cell constituents and it will then be possible to assess more accurately their respective roles, and their connection with growth and metabolism in cancer. The authors' present view is that as a re-

sult of prolonged cell injury by chemical agents, by repeated dosages of x-rays, and by other carcinogenic agents, intercellular wound hormones may be set free over a prolonged period. The best evidence in favour of this view would undoubtedly be the production of malignant tumours by repeated injection of these substances into animals, and experiments on this point are understood to be in progress.

Growth inhibition by carcinogens.—The interesting observation of Haddow (169) that intraperitoneal administration of colloidal carcinogenic agents (1,2,5,6-dibenzanthracene, 1,2-benzpyrene, 5,6-cyclopenteno-1,2-benzanthracene, and 1,2-benzanthracene) markedly inhibits growth of the Jensen rat sarcoma, while the noncarcinogenic anthracene and phenanthrene are without effect, has led to numerous studies of the action of carcinogens on tumour growth. Independently of Haddow's investigations, Morelli & Guastalla (170) have obtained somewhat similar results.

Haddow & Robinson (171) extended the earlier observations to Rous chicken sarcoma and to chemically induced tumours. Definitely carcinogenic compounds and also certain compounds such as chrysene and benzantracene, with very little or no carcinogenic activity, inhibited tumour growth; other related noncarcinogenic compounds had no effect. The estrogenic compound 9,10-dihydroxy-9,10-dipropyl-9,10-dihydro-1,2,5,6-dibenzanthracene was inhibitory, while 1-keto-1,2,3,4-tetrahydrophenanthrene was not. The growth rate of spontaneous mouse tumours was similarly affected (172, 173). A prolonged inhibition of growth followed the administration of the carcinogenic substances 1,2,5,6-dibenzanthracene, 1,2,5,6-dibenzacridine, and styryl 430, while noncarcinogenic substances (pyrene, 1,2,3,4-dibenzanthracene) gave either a transient interference or none at all. Primary, chemically-induced sarcomas were less susceptible to the inhibitory action of carcinogenic agents than were spontaneous or transplanted tumours (174). There was no specificity in this towards the actual substance which induced the tumours, since other similar compounds possessed virtually the same activity. Later and more comprehensive researches (175) showed that tumour growth was inhibited in 86.5 per cent of 171 experiments with thirty-four carcinogenic chemicals, while in the case of thirty-four noncarcinogenic, but related, compounds there was absence of any inhibitory effect in 79.6 per cent of 79 experiments. It was concluded that carcinogenicity and growth inhibiting properties are closely associated. Other workers who have repeated these experiments have mostly obtained similar though usu-

ally somewhat less striking results, perhaps because of the narrow margin between inhibitory and toxic doses (176 to 185). On the whole these workers followed the technique of Haddow and co-workers, but Bauer (181) treated twenty-two human cases of cancer by intratumoural injection or superficial application of 3,4-benzpyrene. Seven of these cases remained healed for over two years. Turner (185) made comparative tests of seventy-five phenanthrene derivatives (including colchicine) on mouse sarcoma 37. The most effective inhibitors were 9-aminomethylphenanthrene hydrochloride, dihydrothebainone hydrochloride, and dihydro-de-N-methyl dihydrothebainone. Franks & Creech (186) have attempted the preparation of antigens by combination of carcinogenic hydrocarbons with carbamido-casein.

Workers who failed to observe the effects claimed by Haddow include Appel *et al.* (187) who found more rapid growth and metastasis of the Brown-Pearce rabbit carcinoma in animals injected with dibenzanthracene. Alapy (188) found that rats were insensitive to prophylactic doses of hydrocarbons which retarded tumour growth in mice, and Rarei & Gummel (189) also failed to detect any inhibitory effect of 3,4-benzpyrene on the growth of rat tumours.

Many of the above investigators (171, 172), observed that carcinogenic compounds and, to a less extent noncarcinogenic compounds, which inhibit tumour growth, also inhibit the general body growth of young experimental animals. Neither type of growth inhibition is proportional to carcinogenic activity, as tested in the usual way on the skin of mice. As inhibitors of rat growth, the activity of the compounds decreases in the order 1,2,5,6-dibenzacridine, 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, which is the reverse of the carcinogenic order. The inhibition of body growth is very prolonged, or permanent, and it seems probable that the growth inhibition produced by these substances is not tumour specific.

The inhibition of growth of young rats caused by feeding methylcholanthrene, benzpyrene, and also by the noncarcinogenic hydrocarbon pyrene, is abolished if *l*-cystine or *dl*-methionine is added to the diet (190, 191). Injections of glutathione act similarly. These observations suggest a relationship with the essential sulphur-containing amino acids, for other substances (glycine, taurine, inorganic sulphate) exerted no protective action on body growth. It is probable that a deficiency of the sulphur-containing amino acids arises through the efforts of the body to detoxicate the carcinogenic and other hydrocarbons. It is known (192)

that this is one of the routes by which detoxication of anthracene may occur, because anthrylmercapturic acid appears in the urine of rats and rabbits which are fed on a diet containing anthracene. Similar proof in respect to the other hydrocarbons mentioned is not yet available.

Natural oil of wintergreen, heptaldehyde, methyl salicylate, etc.—In a series of papers, Strong has presented data indicating that substances related to natural oil of wintergreen (*Gaultheria*), may, when administered to susceptible strains of mice, considerably modify the course of development of mammary cancers. The natural oil, given in the diet, may delay the onset of spontaneous cancer (193) and a cumulative effect was observed after several weeks of treatment (194). Not only did the treated mice live longer after the development of mammary carcinoma, but the longer the treatment was continued the longer the survival period (195). Oil of wintergreen in a mixture of components, the main one (95 per cent) being methyl salicylate. This pure synthetic substance had very little or no effect, however, (196) when given to mice daily after the onset of cancer. On the other hand, the natural oil, administered to mice of the Strong A strain at the rate of one drop per 2 gms. of the standard oatmeal diet, caused darkening, increase of connective tissue, spontaneous haemorrhage and liquefaction of many of the tumours, in some cases followed by complete regression (196); the survival time was not, however, increased.

Further study of the fractions obtained by distillation of the natural oil, showed that the high-boiling portion was ineffective while the low-boiling one caused regression of the tumour in four out of thirty-four animals, slowed the growth rate, and increased the survival time (198). Thus it appeared that some trace of a substance of low-boiling point was the active constituent of the natural oil. Since heptaldehyde was an ingredient of the low-boiling fraction, mice were put on a diet containing this substance, and in six out of twenty-five mice there occurred a very pronounced softening and liquefaction, with complete regression of the tumour (199). Also, injection of heptaldehyde (0.1 to 1.0 cc.) into ten dogs with spontaneous tumours caused softening, which was accompanied in "several dogs" by the complete regression of the tumour (200). Added to the diet, heptaldehyde influenced small spontaneous tumours of mice to a greater extent than larger ones (201). Since it appeared that more of the pure heptaldehyde was required than of the crude low-boiling fraction of oil of *Gaultheria*, the author has suggested a synergistic effect of heptal-

dehyde and methyl salicylate, and has successfully employed a mixture of three parts of heptaldehyde to one part of methyl salicylate (202).

These results have not found general acceptance. Baumann, Kline & Rusch (203) found that addition of heptaldehyde to the diet had no effect on the growth of spontaneous or induced mouse cancer. Pybus & Miller (204) observed no influence whatever on a series of forty-six mice of the Simpson and hybrid strains which bore spontaneous tumours, compared with a similar number of control animals.

Boyland & Mawson (205) consider that heptaldehyde inhibited the growth of spontaneous mammary tumours of three mice (excluding one which died within a few days) with partial regression in one mouse, but this result is far from convincing. Heptaldehyde had no effect on grafted tumours. Other aldehydes, ketones, and glucosides were tested by these authors and the only other active substance was citral, which caused some inhibition of growth of both grafted and spontaneous tumours when given orally. Willmer & Wallersteiner (206) report that the growth and cellular activity of *in vitro* cell cultures, including those of a dibenzanthracene chicken sarcoma, were inhibited by aldehydes, notably glyceraldehyde, methyl glyoxal, propionaldehyde, butyraldehyde, and benzaldehyde, but not by the corresponding acids.

The points of special interest in the work described above are that the substances are stated to be active when given orally, and that spontaneous rather than grafted tumours are affected. The converse of this appears to apply to most other inhibitory agents, such as bacterial filtrates (207) or colchicine (145, 208), though Amoroso (209) has reported effective treatment of a spontaneous tumour of a dog by this substance. Boyland (210) has found that the growth of both types of tumour is inhibited by 4,4'-diamino-diphenylsulphoxide and sodium sulphanilyl-sulphanilate given orally.

Roffo (211) has observed the regression of a spindle-cell carcinoma and of adenocarcinoma in rats following either intratumoural or remote injection of an acid hydrolysate of ox heart muscle. Similarly, autolysates and hydrolysates of heart muscle inhibit the growth *in vitro* of cells from spindle-cell sarcoma but not that of fibroblasts.

HORMONES AND CANCER

Investigations during the past five years have consisted mainly of an intensive study of the effect of hormones on the production of malignant tissues in the mammary gland of susceptible mice. It will

be remembered that this action of estrone was first shown some years ago by Lacassagne (212) who maintained that in the first instance malignant changes could only be produced in the mammae of animals with a spontaneous incidence of this disease. In other words, treatment with estrone increased the incidence over and above the spontaneous incidence. Later on, this view has given place to the one that, provided enough estrone be given over a long enough period, it is possible to induce malignant changes in the mammae of any mouse. The question has arisen as to whether this action of estrone is due to the direct carcinogenic action or whether it is due to the exercise of its physiological function under abnormal conditions.

The supporters of the previous view have likened the structure of estrone to that of the polycyclic carcinogenic hydrocarbons, and have claimed that its action at a distance is the same as the local action of the carcinogenic hydrocarbons. One of us (213) has pointed out that the actions must be considered as being in two entirely different classes. In this communication attention was called to the fact that estrone tested by the same methods as those employed in examining the carcinogenic hydrocarbons proves to be entirely inactive when painted on the skin. One must conclude, therefore, that the mechanism of the production of carcinoma by the carcinogenic hydrocarbons and by estrone is entirely different, and in referring to estrone as a carcinogenic substance a very strict distinction must be maintained between its action and that of the carcinogenic hydrocarbons. It has now been possible to rule out completely the suggestion that estrone acts through its polycyclic structure. The synthesis of stilbestrol, 4,4'-dihydroxy- α,β -diethylstilbene (214) provides an estrogenic substance of more power than estrone, which completely lacks the polycyclic structure.

This substance has been the subject of intensive research in relation to its power to produce carcinoma. When painted on the skin it is completely without action, and in this respect resembles the properties of estrone. When, however, it is administered to mice in the same way as estrone for the purpose of producing carcinoma of the mammae, it has been found to be quickly successful. Thus Lacassagne has reported (215) the production of carcinomata in the mouse by the use of this substance. Similar results have also been found by Bonser *et al.* (216) with triphenylethylene.

During the last year evidence has been produced that estrone or an estrogenic substance is not the only factor necessary for the pro-

duction of cancer. The investigations of Cramer & Horning have led these workers to consider the evidence of experiments on various animals such as the rat and mouse, and to assume that there must be a factor inherent in the animal which they term "susceptibility" (217). They showed that the high incidence of mammary cancer in the susceptible strain of mice can be prevented by the injection of the thyrotropic hormone. They also claimed that by the injection of this hormone it was possible to reduce the extensive enlargement of the anterior lobe of the pituitary which occurs in males after prolonged treatment with estrone, and they concluded that the production of mammary cancer is extremely complex, producing at first changes in many of the endocrine glands, with the production of "endocrine imbalance."

At a later date (218) they also demonstrated certain changes in the adrenal gland of animals treated with estrone. They claimed that adrenalectomy is followed by very direct changes in the thymus, the pituitary, and the mammae, and that continuous treatment with estrone renders mice resistant to the effects of adrenalectomy and that such animals survive a longer time after operation. Finally, adrenalectomy according to these workers makes mice resistant to the estrogenic hormone. It would appear to diminish the action of the estrogenic hormone on the pituitary, and therefore the secondary effects of the pituitary inhibition on somatic growth, atrophy of thymus, testis, etc., are very much less marked in the adrenalectomised animal. As a result of these experiments Cramer & Horning conclude that the secretions of the adrenal gland are synergic with the estrogenic activity of the ovary.

The same workers later called attention (219) to the relationship between brown degeneration of the adrenal glands and the incidence of mammary cancer in inbred strains of mice. In view of the involved nature of the evidence it is perhaps advisable to quote the conclusions of the authors in their own words:

Our present conception of the significance of the degeneration in the adrenal gland for the aetiology of mammary cancer may be summarized as follows. It is not in itself the cause of mammary cancer, since in the high-cancer strains it develops in practically every mouse, male or female, whether the mamma of these animals is cancerous or not. Nor is the development of cancer in the mamma absolutely dependent on its presence in every case, since in the low-cancer Bagg Albino strain and in the mixed strains mammary cancer may develop while the adrenal is intact. The factors which determine the onset of cancer in the mamma—what we have called the remote causes of cancer—are as

varied as they are for the skin and probably for most other organs or tissues. One of these remote causes is an endocrine imbalance, and the spontaneous development of a degeneration in the adrenal medulla is one of the ways in which such an endocrine imbalance is brought about.

The problem has been very well reviewed by Lacassagne in an article entitled *Relationship of Hormones and Mammary Adenocarcinoma in the Mouse* (220). Lacassagne considers the problem from the same points of view as those stated in the beginning of this section, namely whether the carcinogenic action of estrone is due to direct action on the mammae or to some complex indirect series of actions. As has been said by one of us (213), there is direct evidence against the first assumption in the fact that estrone and estrogenic hormones are not carcinogenic in the same sense as the hydrocarbons of the type of methylcholanthrene and 1,2,5,6-dibenzanthracene. Again, the synthesis by Dodds and co-workers (214) of highly active estrogenic substances without the condensed carbon ring system, such as diethylstilbestrol, and the demonstration that these substances are capable of producing mammary carcinoma in suitable strains of mice would appear to put out of court the possibility of direct action as an explanation of the phenomenon. If we have to accept indirect action, then we must consider various factors that might play a part:

(a) The first suggestion that the pituitary was involved was made by Corner in 1930 (221), after observation of the development and changes in the breast following the injection of anterior pituitary extracts.

Many attempts were made to find out the effect of hypophysectomy on the development of mammary carcinoma in animals receiving estrone injections, but the results were discordant, and probably depended upon whether atrophy of the mammary gland is allowed to occur before the estrone injections are begun. Gomez, Turner & Reece (222) maintained that the phenomenon is not due to synergistic action between the pituitary and ovarian hormones, but that the estrone actually reacts through the pituitary. This result they based on the fact that if they transplanted the hypophysis of untreated animals into control animals no change in the breast occurred. If, however, they transplanted the pituitary gland of animals previously treated with estrone, then breast development took place. As a result of this and other experiments these workers were led to postulate the presence of a "mammmogenic substance" in the pituitary.

(b) The action of estrone on the anterior lobe of the pituitary has

been known since 1929, when Smith & Engle (223) demonstrated the inhibiting effect of such treatment, and there now exists an extensive literature on the subject. The gland enlarges, yet at the same time a decrease in secretion is shown by atrophy of the testis and ovary. On the other hand cytological studies would seem to indicate a marked increase in secretory activities. It was on the basis of these considerations that Cramer & Horning developed the experimental work already referred to. They suggested that strains of mice predisposed to mammary cancer might show an increased susceptibility to the systemic effects of estrone (224), and, as already pointed out, they suggested that this action of estrone by producing imbalance might be responsible for the production of carcinoma of the mammae; in support of this they stated that injections of thyrotropic hormones resulted in inhibition of the carcinogenic effect of estrone.

Lacassagne in his review raises a number of objections to this theory. For example, if the animals are treated with equiline or equine, mammary carcinoma develops in the animals without the occurrence of the severe histological changes in the pituitary gland. Again, the essential action of estrogenic substances is to produce a pituitary inhibition which, like hypophysectomy, leads to atrophy of the mammary gland. There is no correlation between the severity of the anterior lobe changes and the incidence of carcinoma. In a number of animals it is possible to produce the most advanced pituitary changes without producing any changes in the mammae. Asdell & Seidenstein (225) repeated the injection of thyrotropic hormones, and were unable to find an action similar to that described by Cramer & Horning. In the experiments of Lacassagne himself injections of thyrotropic hormones entirely failed to prevent the occurrence of mammary carcinoma in animals treated with estrone (226).

Another argument against the conception of Cramer & Horning is that produced by the experiments of Loeb & Kirtz (227), who showed that if they implanted under the skin of mice less than two months old the pituitary of their brothers or sisters, the gland survived for a period of from eight to twelve months. In virgin females such an operation resulted in marked increase in activity and hypertrophy of the mammary gland. In animals with a high incidence of mammary carcinoma it produced the same effects as estrone. On the other hand, if the animal were castrated prior to the transplantation no changes in the mammary glands were observed. From this the authors conclude that the pituitary must act in co-operation with

the ovaries, and suggest that possibly the corpora lutea might be involved.

It has naturally occurred to experimental workers that if it is possible to produce carcinoma of the mammae with estrogenic substances there might be a possibility of curing this condition with other hormones. One of the first to be experimented with was of course the male sex hormone, and there is an extensive bibliography upon the effect of testosterone on the production of malignant changes by estrone. Lacassagne & Reynaud (228) demonstrated clearly that testosterone administered to young female mice of a highly susceptible cancer strain resulted in a marked fall in the incidence of mammary carcinoma, but at the same time there was an almost complete lack of development of the mammary gland. The question naturally arose as to the mechanism of this inhibition and as to whether it is merely due to lack of development of the breast. This has been the subject of a great deal of work, but at the present time there appears to be no clear-cut answer.

The action of testosterone can be considered under three headings: it prevents the development of the follicles, corpora lutea being absent, and there is atrophy of the interstitial cells; the mammary glands do not develop, and are present only in the most rudimentary form; and finally, the pituitary is very much reduced in size. It would appear from these observations that testosterone injections inhibit the pituitary and also the secretions of the ovary, and that since the mammary gland cannot develop it is not possible for carcinoma to occur.

The effect of testosterone on the mammary glands of adult mice is plainly of importance, and a study of it has been made by Nathanson & Andervont (229). They used a strain with high cancer incidence. The animals had all been pregnant on one occasion, and the litter had been sacrificed after twenty-four hours. At once injections of testosterone propionate were given. By contrast with the controls they showed that testosterone greatly reduced the incidence of carcinoma.

It can be seen that the problem is extremely complicated, and that a great deal more experimental work must be done before the mechanism of the inhibition is understood. In general it would appear that carcinoma can occur only when a certain amount of development of the mammary gland has already taken place, and that this development is dependent upon both the estrogenic and the hypophyseal hormones.

THE VIRUS PROBLEM

During the last ten years there has been an intense attack on the constitution of the active agents responsible for tumour production. The greater part of this work has been conducted with the filtrable tumours of fowls, known as the Rous sarcoma. A general summary of the position up to 1935 will be found in Rous's Harvey Lecture for that year (230). By 1930 it was generally recognized that the active principle of the Rous agent was itself a protein or associated with the protein fraction. This was arrived at by a study of the precipitation reactions, the activity always being precipitated with the proteins.

A long series of papers by Murphy, Claude, and their collaborators from the year 1932, indicate beyond any doubt the correctness of the above statements. They showed that by electrodialysis it was possible to produce a precipitate containing all the activity. Frankel, however, had previously shown it was possible to precipitate activity by bubbling carbon dioxide through a solution, and since during the course of electrodialysis the solution becomes acidified it would appear that herein may lie the explanation (231).

A very large series of investigations was performed by treating the active filtrate with buffers. It was found that the activity could be precipitated by slight acidification and could be redissolved in alkali several times without appreciable loss of potency. Below pH 4, however, activity was lost. Murphy and his collaborators then studied the adsorption on aluminium hydroxide (232). They showed that most of the inactive material was adsorbed on the aluminium hydroxide and that the supernatant fluid contained only 10 per cent of the nitrogen originally present and most of the activity. According to these workers, the supernatant fluid gave no reaction for proteins but gave a positive test for carbohydrates. Moreover, they observed that the activity of the supernatant fluid appeared to be greater than that of the original tumour extract, and this they explained by assuming that they had removed an inhibitory substance. At a later date they were able to prepare specimens of the inhibitory substance by eluting the aluminium hydroxide precipitates by means of basic sodium phosphate. The presence of the inhibitory substance was confirmed by Sittenfield, Johnson & Jobling (233) who succeeded in separating it from the active agent by repeated precipitation at pH 4 and elutions at pH 8.

In view of later work it would appear that the claim that the active supernatant fluid did not contain protein was due to the fact that the solutions were too dilute to respond to protein tests.

Jobling & Sproul (234) maintained that the lipid fraction of the tumour extracts was of very great importance, and was possibly the real active fraction of the agent. The work of Pollard & Amies (235) however, threw such grave doubt on this view that unless further evidence is produced it would appear to be untenable.

One of the most recent papers on the chemical aspect is that by Pollard (236). He describes a combination of a number of methods for the preparation of highly active infective products. Thus by means of ultracentrifugation in a Sharples centrifuge it is possible to effect separation of active material from a large volume of inert material in the extract. The material can further be concentrated by the application of physicochemical methods such as the adsorption of infective material on alumina as described by Claude (237). Alternative methods consist of digestion of the extract with trypsin, in an attempt to remove inactive material. No matter how active the preparations proved to be when obtained by the application of the above types of methods, it was always found that they contained small quantities of lipid substances. Very prolonged extraction fails to remove the whole of the lipid and up to the present no lipid-free agent has been found. It would appear possible that the lipid plays an essential part in stabilizing the suspension and is responsible in some way for holding the active protein in an active state.

It will be remembered that in the case of the crystalline tobacco mosaic virus, Ross & Stanley (238) showed that its activity was associated with the number of free amino groups, because treatment with formaldehyde resulted in diminution in activity, whereas removal of the combined formaldehyde by dialysis and other methods resulted in the restitution of activity. Attempts to repeat this experiment with the Rous agent, however, have proved to be unsatisfactory.

The application of the ultracentrifugation method to the study of viruses in relationship to malignant tumours has been confined entirely to the Shope rabbit papilloma virus. Beard & Wyckoff (239) were able to isolate a homogeneous heavy protein which was capable of reproducing all the activities of the extract, and in fact represented a concentration of many thousand times the activity of the original material.

It is not proposed to review here the work on the crystalline virus

since this is obviously outside the terms of the review. A very full review of the various aspects of plant and animal viruses will be found in the volume entitled, *Handbuch der Virusforschung* (243).

It would appear inopportune at the moment even to attempt to summarise the present-day view concerning the relationship of viruses to cancer. A critical review of this subject in which various factors are brought out very clearly will be found in Andrewes's presidential address to the section of comparative medicine at the Royal Society of Medicine (240). As Andrewes points out in order to explain cancer as a virus phenomenon it would appear necessary to postulate a latent virus infection. Great strides have been made by the application of various methods to the study of fowl tumours. Thus normal fowls as they grow older develop antibodies capable of neutralising Rous filtrates. The titre is, however, very much lower than those in bodies infected with slow growing tumours. The question arises as to whether all fowls carry the virus, and does some special form of irritation enable the virus to attack the tissues and produce a tumour. The experiments by McIntosh (241) have a very important bearing on this problem. McIntosh reported that it was possible to produce a tumour by inoculating fowls with tar. In a number of instances these tumours were capable of propagation by cell-free filtrates. This work was extended in 1939 by McIntosh & Selbie who reported the production of tumours capable of transmission by cell-free filtrates (242). These observations would appear to bring together two entirely different, and at first sight, apparently divergent lines of research. The production of tumours by purely chemical agents such as condensed carbon ring compounds has been extensively reviewed in recent years. Others, working with tar-produced tumours, have failed to obtain active filtrates, and at present the reason for this is obscure. Andrewes, however, quotes a very interesting experiment performed by himself in which the tar tumour which failed to be propagated by cell-free filtrates grew vigorously when inoculated into pheasants, which in turn developed active neutralising antibodies against the Rous filtrate in the blood. As Andrewes says, "The suggestion is a strong one that the nonfiltrable sarcoma contains a virus serologically related to Rous virus though not directly demonstrable by filtration experiments."

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PLANT PIGMENTS

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A review on plant pigments occupies a somewhat anomalous position because herein are grouped all plant constituents capable of evoking a certain subjective response to the eye, namely color. In most cases, we do not know whether the absorption of visible radiation—their one common property—is incidental to their main function or not.

Our interest in vegetable coloring matters as agents in the tanning of hide and the dyeing of textiles has declined. A more recent phase centered our interest on the plastid pigments, from which have arisen impressive contributions on chlorophyll, carotenoids and their relation to vitamin A. As our knowledge of the chemical structure of such compounds grows, a third phase is developed, that of evaluating the physiological significance of the pigments and their control by genetic factors. Here we have but tantalizing glimpses of the possibilities. The potentialities of the pigments in problems of hydrogen transfer are obvious. The effect of carotenoids on reproductive processes in lower plants has just been discovered. Co-operation between chemist and geneticist has yielded valuable information on inheritance of anthocyanin pigmentation.

Knowledge of chemical structures is an essential prerequisite which must occupy a prominent position in this review, but where possible, problems confronting the biologist are presented, and progress is evaluated. The review covers the period from 1937 to 1939 except for certain irregularities since September. Pigments of the fungi are covered by Professor Raistrick (*q.v.*).

CHLOROPHYLL

Chloroplasts.—The fragility of chloroplasts has hitherto limited examination to that attainable with the microscope. Procedures are now available for isolating not only chloroplast contents (1) but also substantially intact chloroplasts (2, 3), and the high protein and lipid content is confirmed. It is deduced (2) on the basis of 100,000 mol. wt. for protein that there are thirty chlorophyll molecules per molecule of protein. Copper and iron are concentrated in the plastid (3), but

objection may be made to the deduction magnesium is in inorganic form, as the fractionation with trichloroacetic acid would remove magnesium from chlorophyll. The absorption spectra of individual chloroplasts have been obtained (4). The band at 6800 Å has apparently been resolved into four bands, an observation difficult to evaluate. Chloroplasts are not homogeneous so that there might be greater inherent errors in light-scattering from a single plastid than from a suspension which would interpose a more homogeneous screen in the light path. The situation might be clarified by similar findings on isolated suspensions.

Chloroplastin.—This term is used to designate the pigments in leaves (5). "Phyllochlorin" (6) is inadmissible; it has been pre-empted for a chlorin as the structure and name imply. "Photosynthin" (7) for bacterial pigments may be objected to, on the ground no photosynthetic attributes have been shown for the isolated material. Possibly the term "bacterioplastin" may prove acceptable.¹ A chloroplastin fraction (6) has been isolated from green leaves with aqueous digitalin. It has apparently been denatured (8), which might account for the absorption maximum at 6750 Å instead of 6800 to 6820 Å, the usually reported value. "Bacterioplastin" (photosynthin) from the purple bacteria has been isolated by shattering cells with sound waves of 15,000 to 21,000 cycles per sec. As in the other cases (5, 6) the carotenoids appear to be attached to the same or similar protein molecules from which they separate only on disintegration of the whole complex. An undenatured chloroplastin (5) in water at pH 7.2 to 7.4 is stable to light and oxygen, when held at 0 to 2°. Absorption and fluorescence are those of the living leaf. On drying, or with change in pH or temperature, the protein is denatured. Ratios of the individual pigments are the same as from regularly extracted leaves. The percentage composition of chloroplastin is about: protein, 80, lipid, 10 to 20, and pigment, 5 per cent. This is possibly in as fair agreement with analyses of the whole plastid (2, 3) as may yet be expected.

Structural chemistry

These notes may clarify the situation with respect to chlorophyll structure for the reader not conversant with reviews (5, 9, 10, 11). There are two important groups of porphyrins derived from chlorophyll: (a) Porphyrins resulting

¹ Terms such as thioplastin, purpuroplastin, verdoplastin could be added, when needed.

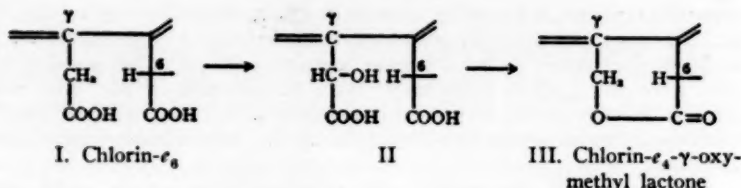
from mild reduction, followed by reoxidation in air, retain the isocyclic ring intact. The key position held by pheoporphyrin-*a*₈ isomeric with pheophorbide-*a* is repeatedly emphasized (5, 10) and must be appreciated. (b) The second group arises from drastic alkali degradation. Here the nomenclature may offer difficulty. It arose from the necessity of describing a compound with incomplete knowledge of its structure, hence the use of Roman and ordinary numerals and subscripts.

In etioporphyrin, the porphyrin ring comprises four pyrrole nuclei with β -methyl β' -ethyl substitution. Numbering the β and β' positions consecutively (Figure VIa) we see four possible isomers. One may have 1,3,5,7-tetramethyl-2,4,6,8-tetraethyl porphyrin, or etioporphyrin I. Let the pyrrole ring IV be rotated through 180° so that β coincides with the β' position, and 1,3,5,8-tetramethyl substitution is found, system III, to which both chlorophyll and hemin belong. The other two isomers, not found in nature, require rotation of the pyrrole nuclei II and III, and II and IV, respectively.

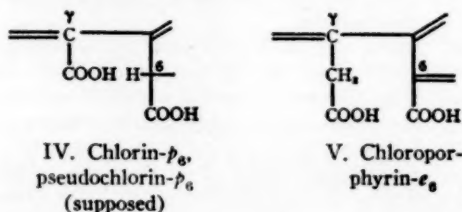
From these four systems may be derived eight pyrroetioporphyrins (tetramethyl-triethyl porphyrin). Systems I and II each provide one; system III provides four (numbered III to VI); and system IV, two (VII to VIII). By methyl substitution on any one of the four methine bridges we have thirty-two phylloetioporphyrins (of which XV is of importance, γ -methyl-6-desethyl). Finally introducing a propionic group in place of one of the three remaining ethyl groups, ninety-six phylloporphyrins become possible (10, p. 46). The synthesis therefore of pyrro- and phylloporphyrins identical with those arising from chlorophyll degradation established the same β substitutions as in system III.

Subscripts refer to the number of oxygen atoms, e.g., chlorin-*e*₆, rhodin-*g*₆. Numerals in ordinary type after a name refer to the strength of hydrochloric acid (expressed in percentage) necessary to extract two thirds of the compound from an equal volume of ether solution, e.g., purpurin 7, purpurin 18. It should be emphasized that phorbin refers not merely to dihydroporphyrin (5) but to dihydroporphyrin with an isocyclic ring (11).

We may next ask why the necessity has arisen for repeated revision of the structure. A partial answer lies in the inadequacy of analytical results to distinguish with certainty a $-\text{CH}_2$ difference in compounds of carbon, hydrogen, oxygen, and nitrogen with a molecular weight of 500. Error may defy detection in the early stages. The most recent modification is a case in point, involving the two hydrogens of the dihydroporphyrin, which are now moved from positions 5 and 6 to 7 and 8. The first intimation (12) came from the finding that the esters of two pyrrochlorin lactones (γ -6) were identical (III), derived from chlorin-*e*₆ (I) and pseudochlorin-*p*₆ (IV). This had far-reaching consequences. Depending on the decarboxylation of the intermediate II, either the lactone or an unstable chlorin is formed. The latter gives rise to purpurin-5. This therefore presents no difficulty.

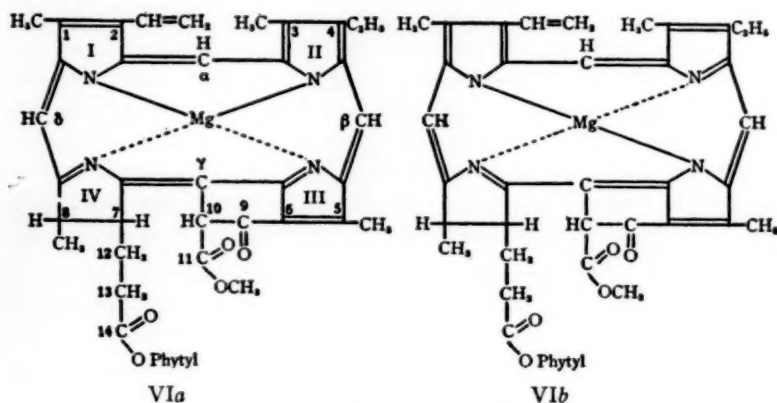


The case for pseudochlorin- p_8 became correspondingly less clear. It depends for its existence on the hydrogen iodide reaction involving the formation of an isorhodoporphyrin- γ -carboxylic acid, virtually identical with chloroporphyrin- e_8 , V.



A higher carbon content was obtained for numerous derivatives than previous analyses had shown, and spectroscopic differences could be reconciled by the assumption of a 10 per cent impurity. The evidence therefore points to the identity of chlorin- e_8 and pseudochlorin- p_8 . Though not optical antipodes (9), chlorin- p_8 and pseudochlorin- p_8 differ in optical activity. Conant's chlorin- f ($=\gamma$ CH —) was optically active, so an additional center of asymmetry had to be found, not on the isocyclic ring. If the argument for the chlorin- p_8 and pseudochlorin- p_8 structures is not tenable—it was viewed by Steele with misgiving (9)—the case for asymmetry in positions 5 and 6 falls. It was then recalled that hematinic acid (β -methyl- β' -propionic maleic imide) cannot be obtained by chromic-sulfuric acid oxidation from chlorins or phorbides, whereas from both blood and leaf porphyrins, good yields are obtained. Neither rhodins nor phorbides of the b series with a 2-vinyl substitution yield methylethylmaleic imide in the basic fraction of the oxidative breakdown products. Now from deuterohemin or any β -methyl substituted nucleus with a free β' position, citraconimide is obtained (methylmaleic imide). Phyllochlorin (1,3,5,8-tetramethyl-2-vinyl-4-ethyl-7-propionic acid- γ -methyl chlorin) on oxidation gave citraconimide as position 6 is free, but no hematinic acid. These facts are explained by placing the two hydrogen atoms

in positions 7 and 8. Two formulae VIa, b are offered (13). Revision of formulae previously listed (11) is simply made by transference of the two hydrogen atoms to positions 7 and 8. The same transfer is made for dehydrobacteriochlorophyll. Bacteriochlorophyll on losing phytol and two hydrogens yields 2-desvinyl-2-acetyl-pheophorbide *a* (14). In the original tetrahydroporphin structure of bacteriochlorophyll, there are two more hydrogen atoms to be located, for which positions 3 and 4, ring II are chosen (15).



Alternative structures for chlorophyll-*a*




The reviewer's most serious objection is the attempt to fix the double bonding in the molecule. One must endorse the words of Stoll and Wiedemann (5): "Demgegenüber will die Betrachtungsweise der Physiker die Lage der Doppelbindungen nicht fixieren, und die einzelnen Pyrrolkerne . . . nicht so scharf von einander unterscheiden." The probability of resonance between pyrrole and pyrrolenine ring forms VII excludes the establishment of either as fixed for a par-



VII

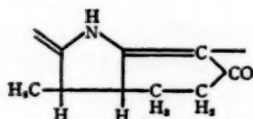
ticular ring. This is supported by x-ray studies on the related phthalocyanines (16) where it is shown the metallic substituent is equidis-

tant from the four nitrogens. Except insofar as we need to express a structure in some mesomeric form, it becomes a matter of indifference

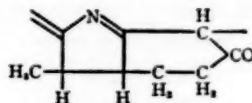
whether we write  or  or even .

If we insist on one, we deny the concept of resonance.

A further difficulty which Fischer has to explain according to his concept of fixed bonds is found in the synthetic rhodins VIII and verdins IX (17). Porphin propionic acids heated with oleum split off water, and give a 6-ring system from γ to position 7 through the propionic carbons 12, 13, 14 on the side chains. These are the synthetic rhodins. From mesoporphyrin (6,7-dipropionic acid) was obtained mesorhodin which could be broken down to pyrroporphyrin-IX (6-propionic acid, 7-H- γ -H), via isochloroporphyrin- e_s . Unfortunately two mesorhodins were obtained, and two mesoverdins. The verdins, in contrast to all other porphyrins have only one absorption band in the visible, *ca.*, 7000 Å. The conversion is explained by isomerization (18):



VIII. Rhodin



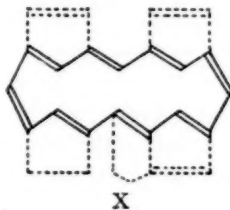
IX. Verdin

A synthetic symmetrically substituted compound was found (16) in 2,3,5,8-tetramethyl-1, 4-dipropylporphin-6, 7-dipropionic acid. There is this difference, that the imino hydrogens are on nitrogens of rings II and IV. By ring closure, only one "propyl" rhodin is found. Fischer then considers the possibility that the double bonding is not fixed, but rejects it on these grounds. The copper complex is markedly more stable with respect to verdin formation, and from the mesorhodin complex it was possible to obtain by breaking the ring, the original mesoporphyrin, identical with the natural material. It is regarded as unlikely that two isomers would be present in the natural product, and so the result is viewed as confirmation of the fixing of the double bonds in mesoporphyrin. Since isochloroporphyrin- e_s is always the main oxidation product of the mesorhodin, and rhodo- and pyrroporphyrins also give this synthetic rhodin reaction, the same distribution of double bonds is postulated for these compounds (seen

partially in the rhodin illustration), and hence a preference for formula VIb.

Some interesting syntheses of porphine,² an isoporphine, and their magnesium, copper, and iron complexes are described (19) and here again specific structures are assigned to the different pyrroles.

A proposed chromophoric ring X (20) is an oversimplification. Modified to include transfer of two hydrogens to ring IV, it may be represented thus:



The italicized portion (reviewer's italics) of the following quotation is by no means correct: (20, p. 211) "Pheophorbide-*a* and pheoporphyrin-*a*₅ are isomers differing only in the place of 2 H atoms. Two H atoms coming from some place (in) the pheophorbide-*a* molecule are able to saturate the vinyl group and thus produce the pheoporphyrin-*a*₅ without changing the color. Obviously these 2 H atoms have nothing to do with the chromophoric ring and hence may be placed at carbons 5 and 6" (read 7 and 8 here). The spectra of porphyrins (porphin structure) and chlorins, phorbides, etc. (dihydroporphin structure) are radically different. The statement quoted ignores this profound contribution of the two hydrogens in the dihydro structure, nor does it allow for the substantial effect of a formyl group in position 3. It does, however, neatly solve our uncertainty as to the metallic linkage, and in the metal-free compound, the tendency of the hydrogens in the imino groups to form hydrogen bonds with the "pyrrole-nine" nitrogen would not be unreasonable, and hence it would be a matter of convenience, not necessity, that determined our allocation of the two imino hydrogens.

The following syntheses are of interest:

(a) *γ-6 ring closure*.—These include pheoporphyrin-*a*₅ from isochloroporphyrin-*e*₄, and mesopyropheophorbide-*a* from isochlorin-*e*₄ (21, 22). Ring closure had been shown with chlorin-*e*₆- and rhodin-*g*₇-

² This spelling is not yet official. It may be hoped it will be restricted to porphine. (Editor's note: Phorbide is here written phorbide in conformity with *Chemical Abstracts*.)

trimethyl esters, in pyridine-soda, but the carbmethoxy group (C_{11}) was invariably lost, resulting in the pyropheophorbide. With methyl alcoholic potassium hydroxide in pyridine in nitrogen gas, it was possible to retain the C_{11} , so that the methyl pheophorbides *a* and *b* were obtained (22). This is important in that a compound has been synthesized with a positive phase test. It is optically active though considerably different from that of the native material. (The starting material of course was also optically active.)

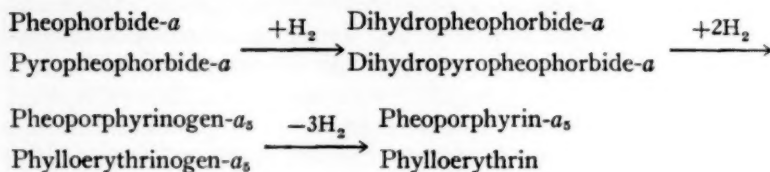
(*b*) *Acetyl and vinyl groups*.—Bacteriochlorophyll-*b* apparently does not occur in nature. The 2-acetyl-rhodin-*g*₇-trimethyl ester was, however, synthesized (15). The steps are similar in the *a* series (14) but hydrogenation of the 2- α -hydroxymeso derivative required more drastic treatment. As Fischer points out, the *b* series reacts more slowly than corresponding members of the *a* series. The 2-acetyl group may be converted to 2-vinyl (14) in 2-acetyl-chlorin-*e*₆-trimethyl ester through partial hydrogenation of its zinc complex. A small yield of 2- α -hydroxy-mesochlorin is obtained. Heated in vacuo at 240°, water is split off, to yield a vinyl group in position 2.

(*c*) *Formic acid iron reaction*.—Methyl pheophorbide-*a* is readily converted to vinyl-pheoporphyrin-*a*₅ by oxidation of the leuco compound formed by the action of iron in formic acid (15). The vinyl-chloroporphyrin has similarly been obtained from chlorin-*e*₆ and by ring closure, vinyl-pheoporphyrin-*a*₅. This is of interest because 2-vinyl-pheoporphyrin-*a*₅ with magnesium and phytol is considered (5) to be protochlorophyll. This derivative has recently been isolated from protochlorophyll from pumpkins (23).

Oxidation of pheophorbide-*a* with silver oxide in glacial acetic acid proceeds with C_{10} , and dehydrogenation of 7 and 8 is effected without changing the vinyl group (24). Similar experiments in pyridine give unstable chlorins which can be esterified to yield the tri-ester of purpurin 7. Oxidation of chlorin-*e*₆-trimethyl ester in propyl alcohol-potassium hydroxide yields purpurins; with silver oxide a 7,8-dihydroxychlorin is formed, from which by ring closure the dihydroxypropheophorbide is obtained. A furane ring postulated for purpurin 7 was abandoned when dihydroxypurpurins were prepared from dihydroxychlorins in potassium hydroxide-propyl alcohol (25).

A new class of purpurins has been prepared (26), the neopurpurins, the reaction being limited to γ -formyl chlorins, and it is assumed aldol condensation takes place between the γ -CHO and C_{12} or C_{13} of the 7-propionic acid side chain.

The status of the leuco-compounds formed as intermediates in the hydrogen-iodide-acetic-acid reaction where phylloerythrin is obtained from pheophorbide-*a* has been clarified (27).



The ease with which the 11-carbomethoxy is split off in reoxidation explains the formation of phylloerythrin in the intestinal tract of ruminants. Leuco-compounds were isolated, and are optically inactive.

Some kinetic studies.—We have referred to Fischer's comment that reactions proceed faster with *a* than with corresponding members of the *b* series. It was pointed out by Joslyn that any structure would have to account for the reactions of the two series, even if kinetic data should not yield clues directly aiding establishment of the correct structure. The simple reaction $\text{chlorophyll} + 2H^+ \longrightarrow \text{Pheophytin} + Mg^{++}$ was studied (28, 29). The reaction is of first order with respect to acid concentration, and chlorophyll-*a* reacts from seven to nine times more rapidly than chlorophyll-*b* (29). Preliminary studies on heats of activation indicate that some difference exists, so it is unlikely the rate differences can be explained purely on the basis of steric hindrance owing to the 3-formyl group in *b*.

More difficult to explain are differences reported in the reaction rates of *a* and *b* with chlorophyllase (30). The ratio 1.8/1 in aqueous acetone has also been obtained in this laboratory (31) and it may be agreed that the rates for decomposition of *a* and *b* are not equal.

It may be possible to explain the increased stability of magnesium in the *b* series on the basis of the 3-formyl group, but in the case of chlorophyllase, we have the breaking of an ester linkage at the end of a 7-propionic acid side-chain. One may possibly conclude that a steric effect permits a more effective union of enzyme and substrate in the case of chlorophyll-*a*.

Properties of monolayers.—Monolayers of porphyrins, chlorophylls-*a* and -*b*, and phthalocyanines were examined on aqueous substrates (32). Owing to the presence of phytol, chlorophyll films (liquid-expanded type) were obtained but could be condensed by pressure to the state of the others (solid-condensed). The metal has

a pronounced effect on the moment, but the phytol governs the intermolecular attractions and adhesion of the molecules to the aqueous substrate. Chlorophyll-*b* with one more polar group has a lower moment, indicating that its moment acts in the opposite sense to the other polar groups. In experiments on the spreading of chlorophyll on water surfaces (20) the monolayer is considered to be solid (p. 203). This lengthy paper contains many statements with which the reviewer flatly disagrees, but withal, it represents an interesting attempt by observation on these monolayers to deduce their condition in the chloroplast and thereby attack the problem of photosynthesis. The compressibility of the chlorophyll film is calculated and is substantially greater at pH 7.6 than at pH 5.4.

Hanson rejects reorientation of the film and considers the change to be caused by hydration. Specifically he regards the isocyclic ring with its enolizable carbon 9 as the seat of this, to be influenced also by the character of the central metal. He states that the formyl group increases the hydrophilic character, yet it is chlorophyll-*a* which clings so tenaciously to the $1/2$ H_2O . Suggestions have been made that the magnesium bonding is more truly covalent in *b*, and to some slight extent of a more ionic character in *a*, which would explain the behavior to acids, and solubilities. Thus chlorophyll-*b* is much more easily and completely thrown out of petroleum ether-acetone solution as the acetone is washed out with water, than is chlorophyll-*a*. However there is no evidence as yet supporting such an interpretation on the basis of exchange experiments with radioactive magnesium.

Amorphous chlorophyll (20) yields an ill-defined x-ray pattern with a spacing of 4.2 Å. The reviewer had considered a similar result to be due to xanthophyll contaminant, but Hanson is more likely correct in regarding this as a certain though imperfect orientation. His opinion is enhanced by finding a spacing of 3.87 Å for ethyl chlorophyllide. He observed a spreading area for the ethyl chlorophyllide monolayer at pH 7.6 of 70 Å², at pH 5.4 of 66 Å². The phytol prevents a perfect crystalline arrangement, but it is supposed that a similar parallel association exists, also inclined 35° from the vertical in the chlorophyll monolayer itself.

The pure chlorophylls.—There seems no reasonable doubt (5) of the claim the two components were first prepared pure by Willstätter. There can be even less doubt as to the inadequate description of at least four out of five attempts to define their absorption spectra because the five are mutually incompatible (33). The suggestion that

acetone extracts contain a "native" chlorophyll (34) which passes over to the "pure" chlorophylls as defined by Willstätter and Stoll is discounted (33) on the basis of inadequate evaluation of the "pure" standards. It cannot be agreed that chromatographic purification produces an allomerized product (20, p. 194) though the possibility of this must always be guarded against.

Physiological studies.—For the most part, these have centered around the problem of variation in the ratios of chlorophyll-*a* to -*b*, and chlorophyll to carotenoids under different environmental conditions. In a number of leaves from *Sambucus*, *Aesculus*, *Fagus*, *Phaseolus*, there was an average decrease of 37 per cent (35) in the *a/b* ratio as between sun and shade leaves. On the basis of such variations, Seybold & Egle attempt to distinguish genetic, time, light, temperature and other "components." The quality of light on the floor of a deciduous forest varies with the season, as shown by the following table (36). In summer conditions, in green-shaded and underwater

PERCENTAGE REDUCTION IN LIGHT INTENSITY
ON FLOOR OF FOREST

Region	March	June
710 mμ	61	14
655	54	3.8
570	51	4.7
520	48	4.3
450	46	3.0

plants, where there is proportionately more chlorophyll-*b*, a more efficient absorption of light is attained. Submerged higher green plants and green seaweeds have about the same *a/b* ratio, of 2.5 (37). The phaeo-, rhodo- and cyanophyceae and diatoms examined contain only chlorophyll-*a*.

Strott (38) observed that a reduction in light intensity had an equal effect on all pigments. Where so many variable factors exist, it is somewhat difficult to make exact comparisons. He noted that with etiolated germinating plants, pigment formation was greatest in the red, followed by the green, and then the blue. The ratios of chlorophyll-*a* to -*b* and chlorophyll to carotenoid were constant. The effect of temperature was to raise the value of *a/b* owing to a diminution in the quantity of *b*. Contradicting part of this (39), chlorophyll-*a* is formed more rapidly in white light than *b*, in etiolated seedlings. If green seedlings are placed in the dark, the *a/b* ratio is increased. A

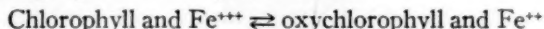
"small but consistent" lowering of the proportion of chlorophyll-*a* to -*b* is also found (40) in wheat plants grown in light from which the blue has been removed. In plants grown under field conditions (41), there is considerable variation in the difference in absorption value for 6650 and 6400 Å in different species. This is explained in certain cases by variation as between species in the *a* to *b* ratio. For a given plant under these conditions, there was virtually no variation. While therefore a genetic "component" with a 3/1 ratio (35) seems somewhat too arbitrary, there can be little doubt that light and temperature may modify the constancy of the ratio obtained under field conditions. Even with the aquatic *Elodea*, such effects have been noted (42). The subject has been reviewed in some detail by Pirson (43). It has been observed, in the dioecious *Salix* and *Populus*, that the chlorophyll content of leaves from female plants is 25 to 30 per cent lower than in leaves from male plants. The difference is lessened when results are expressed on a fresh weight basis (44).

Protochlorophyll.—The isolation of vinyl-pheophorphyrin-*a*₈ from pumpkins has been noted already (23). A protochlorophyll fraction from cucurbit seeds has been shown to contain two green pigments (45). The question is raised as to whether different precursors exist for chlorophylls *a* and *b*. It is interesting to note that protochlorophyll-*b* is the more stable to acid. Seybold & Egle (46) consider that their findings give no support to Noack's views, nor to those of Lubimenko, Monteverde & Liro. The former considered protochlorophyll to be a precursor of chlorophyll, the latter group that it was a post-mortem decomposition product of a chlorophyll precursor. They found, in agreement with Scharfnagel, that chlorophyll was formed by irradiation of only those cucurbit seeds which had no green seed coats. At the same time, both protochlorophylls were found in carotenoid-containing seeds that were turning green prior to illumination. Protochlorophyll-*b* cannot therefore be regarded as a photo-oxidation product of the corresponding *a* component.

The formation and disappearance of chlorophyll in vivo.—The ease with which chlorophyll is formed in the leaf from colorless precursors and its rapid disappearance have rendered studies extremely difficult. Several years ago, Oddo claimed that the role of iron in chlorophyll formation was to be found in the synthesis of pyrrole. Plants fed with pyrrole-carboxylic acid and magnesium but deprived of iron, were able to synthesize chlorophyll. This was questioned by Deuber on the ground that the concentrations used were toxic, and at

lower concentrations no effect was observed. Recently confirmation of the original findings has come (47) from Oddo's group.

To digress, we may expect chlorophyll and phthalocyanines to be treated together from the pedagogic viewpoint by the organic chemist. Let us consider a synthesis of copper phthalocyanine (48): phthalic anhydride, urea, and cupric chloride with catalysts heated under fusion conditions (150 to 200°) give a good yield of pigment. "The ease with which the unusual sixteen-membered ring is formed is startling." *In vitro*, the porphin system has not yet been synthesized so easily. The utilization of pyrrole derivatives by plants should be independently verified. It seems somewhat unimaginative to assume that proline, hydroxyproline, or a pyrrole derivative are *a priori* the probable precursors in the living leaf. There has been no evidence establishing the presence of dipyrrolyl methenes or bilin pigments in higher plants at any stage. To date, S. Aronoff in this laboratory has been unable to detect pyrrole derivatives with at least one free α -position by the isatin method (49) in senescent leaves, which can unequivocally be considered to be derived from chlorophyll. It would be of interest to examine in this connection products of irreversibly oxidized chlorophyll solutions noted by Rabinowitch & Weiss (50) particularly when irradiated by blue light. This paper deals with an apparent reversible bleaching (oxidation) of chlorophyll with certain oxidizing agents. The following equilibrium is postulated:



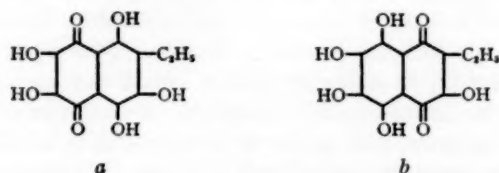
It will not detract from interest in their discovery that many of the remarks on standards (33) are applicable to their spectroscopic data, or that changes in extinction coefficients on standing must be viewed with reserve. The effect of oxygen on the reversibility of the bleaching (51), its acceleration by light, are worthy of note. Since no color change or reduction of ferric iron is observed in the magnesium-free compound, but allomerized solutions give the reaction (50), the phase test mechanism is obviously not involved.

In nature, the destruction of chlorophyll would appear to be photo-oxidative in character, and we do not as yet have evidence suggesting where breakage of the porphin ring takes place.

CAROTENOIDS

The chemistry, structure and distribution of these pigments has been evaluated by previous reviewers in these volumes, also by Bogen (52) and Strain (53).

"*Befruchtungstoffe*."—Of outstanding interest are conception factors which deal with the motility of gametes and conjugation; the attraction of sperm to ovum and conception in the animal world. The pigments concerned are crocetin derivatives in the alga *Chlamydomonas eugametos* (54, 55), and a naphthoquinone in *Arbacia* (56). The latter presents a somewhat simpler case; the pigment echinochrome A is reversibly reducible, of known redox potential, so that the procedure may be governed by a hydrogen transfer mechanism. Kuhn & Wallenfels (56) suggest the role of echinochrome A in relation to sperm may be comparable with that of a coferment in muscle contraction. The eggs only are pigmented, and the echinochrome A was isolated from them. The leuco compound is 2-ethyl-heptahydroxynaphthalene, and the pigment a quinone, probably XI. The



XI. Echinochrome-A (alternative structures)

situation is more complex with *Chlamydomonas*. Moewus (54) observed that *Chlamydomonas*, nonmotile on agar, became motile in water on illumination, or in the dark if supplied with oxygen and a sugar (especially gentiobiose). Without oxygen, motility is obtained if filtrates from motile cells or crocin solutions are added. Crocin, the digentiobiose ester of crocetin, is the motility factor, effective in concentrations of 1 in 250 trillions (250×10^{12}).

Conjugation was observed in motile cells in crocin solution on irradiation with the shorter wave lengths of the visible spectrum. This conception factor was evaluated as follows (54, 55). A precursor, V, formed in red light, on irradiation with the blue passed through two active stages, K_f (female factor), K_m (male) to inactive K_o. V and K_o were identified respectively as the *cis*- and *trans*-forms of crocetin dimethyl ester. The female factor contained approximately three parts *cis* to one part *trans*; the male contained one part of *cis* to three parts of *trans*. Synthetic mixtures behaved in accord with this, and were effective in concentrations of 3×10^{-5} μ g. per cc.

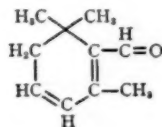
Ambisexuality (also known as relative sexuality) in microorganisms must be briefly considered to clarify the next phase. In certain fungi and algae, numerous strains may apparently be isolated which are capable of behaving as male or female depending on the sex-potentiality of the mate. Such is the case with the mold *Achlya ambisexualis* (57) and *Chlamydomonas*. Moewus (58, 59) differentiates various forms of *C. eugametos* including *f. simplex* and *f. synoica*. The former is dioecious, with separated isogamous male and female gametes. This the reviewer interprets to mean they are heterothallic, and separate male and female hereditary strains can be cultured. Actually eight gametes, female 4 to 1, and male 1 to 4, have been isolated. F^4 is strongly female, F^1 weakly so; the latter is capable of behaving as male in the presence of F^4 or F^3 (F, M are female and male "valence" factors). The sexuality is related to the composition of the *cis-trans* crocetin mixture.

Each gamete secretes a specific material effective only for its own group, but by irradiation with blue light, the specificity is changed to another group, depending upon the degree of change in the *cis-trans* mixture.

The *synoica* form is monoecious (mixed sex) (60) and a non-hereditary sex-determinability has been observed. If *synoica* is treated with filtrate from female gametes, all cells are female, if from male gametes, they become male.

The conception factors are named gamones, and those determining sex, termones, hence andro and gyno-termones.

It has been suggested that protocrocin (a normal C_{40} carotenoid) may be broken up to crocin (C_{20} in the carotenoid part) and picrocrocin (C_{10} in ionone ring form). Crocin is the digentiobiose ester of crocetin, and picrocrocin the glucoside of safranal (XII).



XII. Safranal

The following roles are cast for the various breakdown products (60) in their effect on *synoica*:

1. Protocrocin—the common precursor formed in cells in the dark.

2. Crocin—makes male and female cells motile under anaerobic conditions; threshold value, one molecule of crocin per cell!
3. *cis* and *trans*—Crocetin dimethyl esters, the conception factors, or gamones.
4. Gentiobiose—the motility factor under aerobic conditions.
5. Picrocrocin—the gynotermone) makes all the *synoica* cells female.
6. Safranal, the androtermone, makes the cells male.

The threshold value for picrocrocin is 10^6 molecules per cell, for safranal 10.

A few comments must be offered. While it is difficult for the outsider to evaluate the arbitrary sexuality scale (59, p. 49) which correlates remarkably with observed *cis-trans* mixtures, its simplicity may be its salvation in interpreting a biological phenomenon. Biology deals with a degree of organization which cannot always be treated statistically. The threshold concentration of crocin effectiveness, 1 in 250×10^{12} , has no significance, chemically speaking, unless it be that of the infinitely dilute solution.

In the phycomycete *Allomyces*, it was observed (61) that the sexual plants bore orange male and colorless female gametangia. The pigment consisted almost exclusively of γ -carotene. It is commented (62) that coloring in *Neurospora tetrasperma* is linked with sex factors. Races of one sex bear orange conidia, those of the other are pinkish. No doubt many similar instances could be cited, and we may expect explanations along the foregoing lines.

Isomerization of carotenoids.—The discovery of isomers found by chromatographic adsorption raised two important questions, first as to the occurrence *in vivo* of many isolated components, and second as to the structural implications. The former has been clarified largely as a result of the work of Zechmeister, Strain, and Gillam. Carter & Gillam (63) concur in the view (64, 65) that the carotene isomers found by them as a result of adsorption studies are merely separated, not formed by contact with the adsorbent. The equilibrium (carotenoid \rightleftharpoons neocarotenoid^a) is markedly dependent on temperature. The tendency of the natural carotenoid to isomerize is reduced by storage at low temperature. Solid β -carotene is unaffected, the isomerization occurring only in solution. Strain (53) found several pigments, notably lutein and zeaxanthin were affected by heat treatment. Studies

^a Neo- β -carotene is called pseudo- α -carotene on account of its spectroscopic similarity with α -carotene. See also (148) for structural implications.

on the equilibrium were made (64) with lycopene, β -carotene, and cryptoxanthin. Later (66) the effect of iodine on the equilibrium was observed with respect to several more carotenoids. The neocarotenoid is adsorbed below the original compound in the case of hydrocarbons and cryptoxanthin. If there are two hydroxyls, the reverse is true, and two or three additional isomers are to be observed.

Possible configurational differences are then discussed, including *cis-trans* forms. The spectroscopic data on the neo-isomers indicate a shifting of the absorption maxima toward shorter wave lengths. The relation of band maxima to the number of conjugated double bonds is well known from the early work of Kuhn, Brockmann & Hausser. Neo- β -carotene is spectroscopically similar to α -carotene. In formal fashion, β -carotene is represented with eleven double bonds all in direct conjugation, α -carotene, also containing eleven, with only ten in conjugation. The physicist's view (67, 68) is that the more elongated the molecule, the more the intensity should be confined to the long wave length end of the spectrum, hence the more intense the color. "Available evidence supports the theory if it is supposed the polyene chains normally assume the most elongated possible form. When part of the polyene chain is bent into a ring, the intensity is reduced." One other sentence is applicable possibly to the conjugated system in chlorophyll, when attempts are made to fix the position of the double bonds. "For any model, carbon-carbon distances in the conjugated chain should be nearly equal." Comments on a proposed unit cell (69) for β -carotene are offered with some reluctance because only a fraction of similar studies (70) were published, and adequate data cannot be presented here in justice to both viewpoints. The proposed cell has dimensions $a_0 = 7.75$, $b_0 = 9.5$, $c_0 = 25.0$ Å, $\beta = 105^\circ$. It could therefore accommodate a molecule of rather less than 30 Å. With many outside suggestions, the reviewer had calculated a minimum length of 33.5 Å assuming a zig-zag polyene chain, and a carbon-carbon distance of 1.38 Å (cf. Mulliken's chain and C—C 1.39 Å). Rotation and Laue photographs indicated a crystallographic axis vertical to the flat surface of the crystal, with a spacing of 23 Å. Owing to the size of the cell, an unequivocal interpretation of the Laue photographs was not obtained. When $d_{(hkl)}$ values are calculated for the strongly reflecting planes according to Taylor's data (69) e.g., (102; 004; 104) agreement with powder lines (70) is mediocre.

Leaf xanthophylls.—Studies on the preparation and properties of leaf xanthophylls (53) have yielded definitive results. Certain com-

ponents representing a third or more of the total are apparently discarded in the Willstätter-Stoll procedure owing to their high solubility in methanol, either in the course of partitioning between immiscible solvents, or in the mother liquors. They are highly sensitive to air, so that ordinarily they pass undetected. Their properties, however, are notably different, especially in spectral absorption. The most abundant, neoxanthin (not to be confused with neocarotenoid isomers), has absorption maxima at 437, 467, $m\mu$ (cf., lutein 446, 476 $m\mu$), in ethanol. This and similar components may comprise 20 to 30 per cent of the total xanthophyll. Lutein is in all cases the predominating component, but there are small quantities of isolutein, zeaxanthin, and traces of cryptoxanthin. Violaxanthins and flavoxanthins are also found but they differ from those found in flower petals, sometimes in optical activity, and also in position on the adsorption column, though they have similar absorption spectra. The rapidity with which yellow pigments are oxidized in killed etiolated seedlings should be noted; even in killed green leaves, a 20 to 50 per cent loss is found after one hour. The residual yellow pigment is oxidized more slowly. Strain indicates a possibility that the pigments occur in more than one state, but until further evidence is adduced, it seems as plausible that the nature of the coagulum formed by the leaf-killing procedure would regulate the accessibility of oxygen to the pigments. Certainly the ease of extraction of the pigments with a given solvent is definitely associated with the time and temperature of the hot water treatment. The reviewer disagrees with the statement of Willstätter & Stoll (147) that the effect of hot water on a leaf is to cause a bursting of the chloroplast. The clumping of shrunken chloroplasts in a mass of coagulated cell contents may readily be observed under the microscope.

Red yeasts.—Lodder (71) differentiates two families of anasporogenous yeasts (Torulopsidaceae and Rhodotorulaceae) on the basis of the ability of the latter to form carotenoids. The Molisch test is used, whereby characteristic crystals are obtained when a quantity of the organism is allowed to stand in alcoholic potassium hydroxide. In collaboration with E. M. Mrak, the reviewer has had uniformly negative results. The organism *Rhodotorula rubra* resists boiling for several minutes in alcoholic potassium hydroxide, and no pigment is extracted. Similar attempts to obtain a pigment extract failed in van Niel's laboratory. On prolonged standing in alcoholic potassium hydroxide cells bleach slowly. After cautious hydrolysis with 10 per cent hydrochloric acid, the pigment can be extracted with

acetone (isomers may of course be formed by such treatment). It would seem desirable therefore to plasmolyze the cells, to filter the coagulum and apply the sulfuric acid test, to develop the characteristic blueing, or to transfer an acetone extract to petroleum ether and examine spectroscopically.

In these organisms, Lederer (72) found principally an astacin-like pigment, some torulin, and traces of β -carotene. The torulin is in our experience, spectroscopically more similar to the second of two pigments isolated from *Spirillum* (73). Partition experiments are not sufficient to establish its hydrocarbon nature, and in view of its absorption maxima of 495, 523, 562 m μ (74) (cf. 494, 521, 559 m μ , this laboratory), a hydrocarbon is not to be anticipated if it is a C₄₀ compound. In a related species, *R. Sanniei* (74), traces of other pigments are also reported. The three most abundant are the astacin-like pigment, 29000 μ g., torulin, 143 μ g., and β -carotene 10 μ g. per gm. dried yeast. The nutrition of these organisms is of great importance in relation to pigment production (74) and it would seem worth while to enquire whether members of the Torulopsidaceae might not, in a suitable medium also develop similar compounds before this characteristic is accepted too widely for taxonomic purposes.

New carotenoids.—Systematic investigation of a group of plants is most desirable, as in Tischer's continued studies with fresh-water algae (75, 76, 77, 78). This is not always practicable because of localized distribution of some highly pigmented species, but isolated studies are not always compared sufficiently rigorously, analyses are frequently lacking, and we may reasonably suspect on occasion, known carotenoids masked by new names. Hematochrome from *Hematococcus pluvialis* (75) is, in fact euglenarhodon, the principal component in the pigment fraction. Hematoxanthin, with one broad band, maximum at 513 m μ is also reported (75) but is apparently identical with astaxanthin (90). The resting spores of *Hematococcus* (76) have three esters, one of which is the dipalmitate of euglenarhodon. The question as to whether hematoxanthin (epiphasic) is also esterified in the spores is uncertain. The blue-green *Aphanizomenon* (77) consists mainly of β -carotene (100 mg.), aphanin (epiphasic, not saponifiable, C₄₀H₅₄O, 50 mg.), aphanicin (20 mg.), flavacin (traces), and the hypophasic aphanizophyll (16 mg. per kg. of dry alga). Aphanin and aphanizophyll are similar to myxoxanthin and myxoxanthophyll. The properties of these pigments were further investigated (78), and the structure for aphanin established. It has twelve double bonds, forms

an oxime and the carbonyl group is considered to be in the conjugated system. Aphanin and aphanicin have vitamin-A activity (79) and therefore contain one unmodified β -ionone ring. The California poppy yields eschscholtzxanthin, $C_{40}H_{54\pm 2}O_2$ (80) very labile in the presence of oxygen, containing twelve double bonds, and two hydroxyl groups. Gazaniaxanthin from *Gazania rigens*, an African composite, has the formula $C_{40}H_{56}O$ (81) and is spectroscopically similar to γ -carotene. Eloxanthin (82) from *Elodea* is apparently isomeric with flavoxanthin. A somewhat similar carotenoid, not present in green leaves, is to be found in carrot roots (83). Although carotenoid-protein complexes are to some extent assumed in the chloroplastin (*q.v.*), green pigments of the ooverdin-protein type as in lobster eggs (84) have not yet been found in plants.

Miscellaneous.—The step-wise alkaline-permanganate degradation of lycopene has been followed chromatographically with isolation of interesting intermediates; apo-2-lycopinal, apo-2,12-lycopene-dial (bixin dialdehyde) etc., (85). By accurate measurement of the absorption coefficients of a series of esters of a carotenoid, Strain (80) has developed a new method for molecular weight determination. The chromatographic adsorption of small quantities of carotenoids has also received renewed attention (86).

The problem of phototropism in relation to carotenoids has been the subject of considerable work (87). Two maxima are usually found, *ca.*, 445 and 485 m μ . It seems not improbable that carotene is a sensitizer in the photo-oxidation of auxin.

The isolation of five new carotenoids from alfalfa (88) after acid treatment (e.g., 0.025 *N* HCl) or from silage is possibly related to the isomerization already discussed. The carotenoid content of lemons, limes, and grapefruit decreases on degreening with ethylene, whereas that of oranges increases (89).

ANTHOXANTHINS, ANTHOCYANINS AND RELATED COMPOUNDS

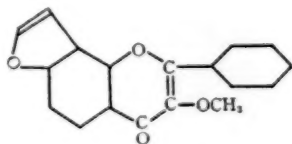
The progressive reduction in the sequence quercetin, cyanidin, and catechin must be familiar to most. We shall not, however, narrow the field to a consideration of flavonols and anthocyanins or we shall fail to consider a wide diversity of essentially closely related structures which do not follow similar sequences. The dihydropyran group, for example, in brazilin or hematoxylin is in essentially the same state of reduction as catechin, but brazilin and hematein are not to be classed as anthocyanins, though analogous brazylum salts may be prepared.

In common with flavonols, coumarins and chromeno-pyrone must be considered as anthoxanthins, in which are included flavanones (dihydroflavone) and chalcones. Rottlerin as an example, has some characteristics of chalcones. All presumably play some part in hydrogen transfer, and this evidence will be considered first.

The production of quinones from *o*-dihydroxy flavones with plant oxidase systems has been noted (91). Quercituron (quercetin and glucuronic acid) the predominant pigment of etiolated leaves (92) may be reversibly oxidized to a quinone. The system tested was ascorbic acid, quercituron, polyphenolase, and oxygen. Anthocyanins may function as hydrogen acceptors, being reduced to catechin-like products (93). Both cyanidin and a catechin condensation product were isolated from autumnal leaves of wild grape. An aldehyde dehydrogenase from horse liver was also employed to carry out dehydrogenation of propylaldehyde in the presence of cyanidin and delphinidin.

The above evidence, limited as it is, represents an important indication of what may be anticipated.

The following compounds have been isolated and the structures established with varying degrees of certainty. Herbacin from cotton flowers is isomeric with quercimetrin, though in behavior it resembles gossypitrin. Herbacetin, 3,5,7,8,4'-pentahydroxyflavone, has been synthesized (94). Tangeretin from *Citrus nobilis* is 3,5,6,7,4'-pentamethoxyflavone (95). Oroxylin from *Oroxylum indicum* is a mixture of baicalein, 6-methyl-baicalein and chrysin. Dikammali gum from *Gardenia gummifera* contains the first derivative of a heptahydroxyflavone found in nature, gardenin, 5-hydroxy-3,6, (or 7)8,3',4', 5'-hexamethoxyflavone (97). Karanjin from *Pongamia glabra* is a furanoflavone of probable structure XIII (98). Three varieties of

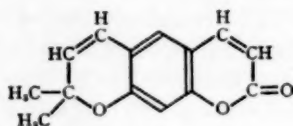


XIII. Karanjin

apples (99) have a quercetin-3-galactoside, and idaein or cyanidin-3-galactoside.

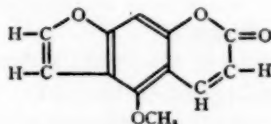
The constituents of *Zanthoxylum americanum* have been examined and xanthyletin and various related compounds have been isolated

(100). They constitute a new type of natural chromeno- α -pyrone XIV. It would take us too far afield to discuss the numerous papers



XIV. Xanthyletin

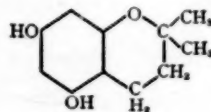
on this phase by Robertson *et al.* (100) or Späth and co-workers (101), but the 2,2-dimethylchromeno ring is common to several naturally occurring coumarins. As a result of studies on apoxanthyletin (102), bergaptene was synthesized. This contains a furanocoumarin ring, XV.



XV. Bergaptene

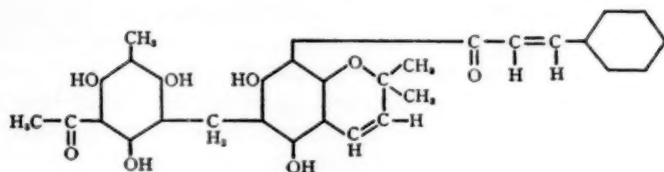
Tambulin has been isolated (126) from *Z. acanthopodium*. It is a 5,7-dihydroxy-3,8,4' or (4',6,8)-trimethoxyflavone, but not identical with tangeretin.

Rottlerin, the coloring matter of kamala, or *Mallotus philippinensis* contains a cinnamyl chromene unit (103). Hydrolytic fission of tetrahydrotrotlerone yielded 5,7-dihydroxy-2,2-dimethylchromane XVI.



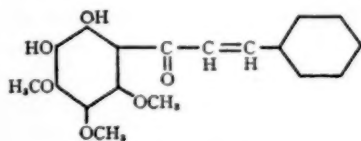
XVI

Brockmann & Maier believe $C_{30}H_{28}O_8$ best fits the formula (104). With alkaline hydrogen peroxide, cinnamic acid was obtained, and benzaldehyde on ozonolysis. This means a benzylidene group and rottlerin therefore has the character of a hydroxy chalcone. Formula XVII is suggested (125). Pedicin XVIII from *Didymocarpus*

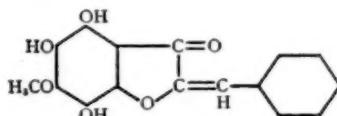


XVII. Rottlerin

pedicellata is a 5,6-dihydroxy-2,3,4-trimethoxy-chalcone, and pedicellin the completely methoxylated derivative (105). Pedicinin XIX is considered to be 3,5,6-trihydroxy-4-methoxy-benzalcoumaranone.



XVIII. Pedicin

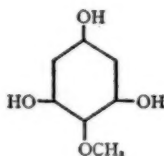


XIX. Pedicinin

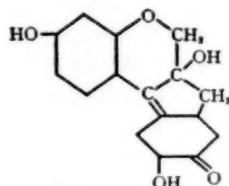
Tectorigenin (106) from *Iris tectorum* yields *p*-hydroxyphenylacetic and formic acids and iretol XX on alkaline decomposition. Since certain coumaranones would also give these degradation products, the compound was re-examined. The resistance of tectorigenin to complete methylation is held to indicate chelation of the hydroxyl group *ortho* to the carbonyl, so the isoflavone structure proposed some years ago by Asahina is favored.

The aglucone of a glucoside in *Murraya exotica* is identified as scopoletin, 6-methoxy-7-hydroxycoumarin (129). Primetin is believed to be a 5,8-dihydroxyflavone (127), with the 4' methoxy derivative in ginkgo leaves.

Difficulties in the determination of the structure of brazilein XXI (107) are that where potential tautomers are immobilized by substitu-



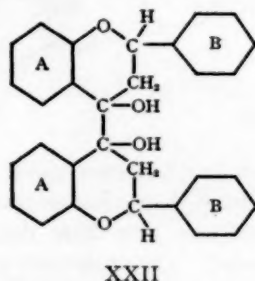
XX



XXI. Brazilein

tion, the structure of the derivative alone is determined with certainty. Methylation studies had been held to favor the 4'-quinone, rather than 7-quinone. It was possible to prepare by hydration of the quinoid a crystalline ethyl ether which yielded a dimethoxy-ethoxy-brazylum sulfate, a pyrylium derivative. Brazylum is thus an indeno-benzpyrylium. The ferrichloride complex has been synthesized. Tetramethoxy-hematoxylin and the corresponding trimethoxybrazilin were oxidized with chromic acid (128) to diketones both of which may be synthesized. The reduction of these diketones has been systematically studied. Results are in accord with present structures for the compounds.

In pursuance of Russell's theory of phlobatannin structure, a number of flavpinacols have been synthesized XXII (108) where ring A may be derived from resorcinol, phloroglucinol or pyrogallol, B from



catechol. Such products are qualitatively indistinguishable from natural phlobatannins. When B is derived from vanillin or *p*-hydroxybenzaldehyde the product is not comparable with the tannin. The flavpinacol obtained by reduction of 2,3'4'-trihydroxychalcone is soluble in water and will tan. The question arises as to whether 3'4'-dihydroxy substitution is essential for tanning. Solubility is influenced more by the number and disposition of hydroxyl groups in B than in A. In the latter, there is a greater effect of hydroxyl on the color. The flavpinacol from quinacetophenone and protocatechual was not distinguishable from hemlock and mimosa tannins (109).

The anthocyanin survey initiated by the Robinsons has been continued (110, 111). A cautionary note (111) indicates that results with autumn leaves are not so conclusive with regard to sugar type. Also where much anthoxanthin is present, it may seriously modify distribution properties and color reactions, and so improved techniques are considered. Leaves may be permanently pigmented with anthocyanin,

they may develop it in the autumn, or they may contain the pigment for a brief period when young. In any event, the vast majority (80 to 95 per cent) contain cyanidin. The distribution of the various types is discussed for leaf, flower and fruit (112). The leuco-anthocyanins are morphologically much more widespread, occurring even in wood and bark. New types in the *Pteridophyta* (ferns) appear to be based on 6-hydroxypelargonidin and 6-hydroxy cyanidin (113). Of the nitrogenous anthocyanins, betanin has been examined (114, 115) in greatest detail. The suggestion is made that betanidin may contain a pentahydroxyflavylium nucleus and ornithine. It is very sensitive to oxygen and contains no methoxy or imino groups. Nudicaulin from *Papaver nudicaule* is a nitrogenous yellow pigment resembling anthocyanins in having apparently the flavylium structure. The name flavocyanin is suggested for the class (130). It is doubted (116) that bacteria contain anthocyanins as many basic dyes would simulate the color reactions of anthocyanins and present evidence is considered inadequate.

The "genetics and chemistry of flower color variation" has been thoroughly worked out for *Lathyrus* (sweet pea) (117). Genetic factors are associated with a difference in the degree of oxidation in the flowers, in pH of cell sap, in relative amounts of anthocyanin and anthoxanthin, and variation in methylation of the anthocyanin is correlated with the quantity of anthoxanthin.

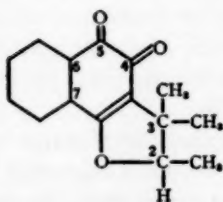
The following miscellaneous items are of interest. The boric acid color reaction of flavone derivatives has been studied (118), no color being obtained with flavanones or fisetin. The importance of 5-hydroxy substitution and the double bond, $\Delta^{2,3}$, is stressed. Absorption spectra of several of these compounds have been reported, and a recent compilation (119) may be cited. Citrin, as vitamin P, is not considered here. It is, however, a mixture of the flavanones, hesperetin and eriodictyol, together with some quercitrin (120).

The biogenesis of anthocyanins may be considered since it has provoked recent discussion. Conceivably anthocyanins may arise from flavonols, leuco-anthocyanins, or catechin-like compounds. In the reviewer's opinion, neither the forceful presentation of Bancroft & Rutzler (121, 122, 123) nor the modification of the Robinsons (124) indicate that the solution is at hand. We must concur in the view that it is idle to speculate on the state of oxidation of leuco-anthocyanins until they have been purified and properly characterized (124). The statement "In leaves, however, it is questionable whether anthocyanin

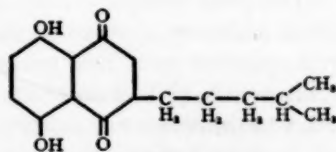
serves any useful function, . . ." (112) is to be deplored. We know that the response of the leaf to certain environmental and possibly also nutritional factors is the prompt formation of anthocyanins, and we admittedly do not know yet what function this serves. The plant may immobilize what it cannot excrete, for example, calcium oxalate, but there is no indication anthocyanins are immobilized in young or permanently pigmented leaves, nor even in autumnal reddening, until death ensues.

The problem then is not a matter of whether suitably prepared extracts of leaves may undergo certain reactions *in vitro*, because the results can at best be suggestive of possibilities *in vivo*. Buffer systems in the leaf do not permit the pH to fluctuate in uncontrolled fashion, and it is reasonable to suppose that different poisoning systems control the oxidation-reduction mechanism during the life of the leaf under the widely differing conditions of spring, summer, and autumn. Our evidence for anthoxanthins and similar pigments in this role is at present meager, but it is tacitly implied in the suggestion that in some cases flavonols may be reduced, and that in others leuco-anthocyanins may require partial oxidation, when such compounds are considered possible precursors of anthocyanins *in vivo*.

Dunnione (131) from leaves of *Streptocarpus Dunnii* has the formula XXIII, $C_{15}H_{14}O_8$, crystallizing in orange red needles. It is 2,3,3-trimethyl-6,7-benzocoumarane-4,5-quinone. Among other naphthoquinone pigments may be listed lomatiol from *Lomatia* sp. (132) and of course phthiocol and its vitamin-K derivative. The absorption spectra of several naphthoquinones have been reported (133). Alkannan,



XXIII. Dunnione



XXIV. Alkannan

XXIV, has now been synthesized (134). Alkannin has the side chain, $-\text{CHOH} \cdot \text{CH}_2 \cdot \text{CH} : \text{C} \cdot (\text{CH}_3)_2$. Populnin, from Indian tulips, contains glucose and populnetin, a tetrahydroxyanthraquinone (135). The primverosides of purpurin-3-carboxylic acid and rubiadin have

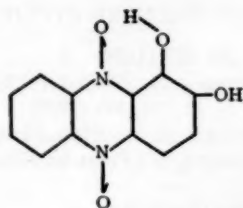
been found in species of *Galium* and *Rubia* (136). Ustilaginoidin is a red anthraquinone pigment from rust (*Ustilago* sp.) (137).

Of considerable interest is a group of pigments of size C_{80} to C_{32} . In one case (the fungus *Penicillioptis*) β -methylantracene is obtained on reduction.⁴ The pigment hypericin belongs in this group. Isolated from *Hypericum perforatum*, it can be resolved into several components chromatographically. The weed when ingested by animals causes them to become sensitive to light striking the white or unpigmented areas of the body. Rigorous proof of the causative agent is rarely offered, but this has been obtained with certainty for hypericin in the past year (138). Other instances are to be found (139, 140).

The absorption spectrum and solubilities of dracorubin, $C_{32}H_{24}O_5$ from dragon's blood, *Sanguis draconis*, have been determined (141). Acetophenone and benzoic acid are obtained on alkaline fusion.

SOME PHENAZINE AND OTHER BACTERIAL PIGMENTS

So far, three phenazine pigments have been isolated from bacteria: pyocyanine, chlororaphine, and a dioxide of dihydroxyphenazine XXV (142) from *Chromobacterium indium*. It is reversibly



XXV. Dihydroxy phenazinedioxide

oxidized and reduced with some difficulty. The phenazines may act as oxygen carriers in the hexose monophosphate system. Certain of

⁴Dr. A. E. Oxford has kindly communicated to us the suggestion that penicillioptin may be a dimer of emodin anthranol. No derivative of emodin was obtained from oxypenicillioptin or the irradiated product. The last-mentioned is almost identical with hypericin. The suggestion (146) that hypericin is a polyhydroxy *meso*-dianthrone, $C_{30}H_{18}O_8$, is viewed with some doubt both by him and ourselves. Our zinc dust reduction product is a strongly blue-fluorescing oil, of mol. wt. ca. 330. Further condensation seems possible, e.g., at one or both *peri*-positions. In such a case, this would yield helianthrone or *meso*-naphthadanthrone. We believe a di- or tetrahydro-structure to be involved, on the basis of our analytical data and in view of the absorption spectra.

this group resemble flavins in the ease with which semiquinones are formed (143).

Except for the green and purple bacteria, it is apparently characteristic of bacterial pigments that they are not essential for normal development (144, 145), and there are striking differences in the nutritional requirements for pigment production. *Pseudomonas Beijerinckii*, causing purple coloration of salted beans requires a bean extract or *i*-inositol to form pigment. The latter is a calcium salt of tetrahydroxyquinone (144).

Traces of copper, on the other hand, are essential to growth in *Aspergillus niger*, and for normal development of black spores, while in *Azotobacter* lack of copper is evidenced only by the absence of the usual brown coloration (145).

Important omissions are those topics which may be considered in other sections under photosynthesis, enzymes, or vitamins at such times as these are reviewed. The reviewer has gained much from numerous colleagues and he is particularly indebted to Professor W. V. Cruess for careful perusal of the manuscript.

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ASPECTS OF INORGANIC METABOLISM IN PLANTS

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The invitation issued to the writer by the editors of the Annual Review of Biochemistry to prepare a review on what they termed Mineral Nutrition of Plants imposes a pleasant if somewhat arduous duty upon me. Its arduousness is all too evident if one considers the vast and rapidly growing literature on the subject (which I prefer to designate as above indicated) even though only the years 1938 and 1939 were assigned for review. Its pleasantness to the reviewer is accounted for by the fact that having been one of the pioneer investigators in one part of this field in the United States he feels a sense of real attachment thereto and an ever-fresh interest therein.

From the numerous subjects to which researches have been devoted in the field represented by the title of this review, I have selected a few for brief discussion since obviously space obliges drastic limitation somewhere in such a review. To these selected subjects I shall now address myself.

THE ESSENTIAL CHEMICAL ELEMENTS

It is nothing short of amazing that after lying fallow for about half a century and mostly so for over a century, this subject is enjoying a recrudescence of interest and activity which few if any would have predicted at the turn of the present century. To a list of ten essential elements recognized about 1920, several have been added so that when the last review of the subject was made in this medium, the total number of elements generally recognized as essential to plants was raised to fifteen; in addition one element, gallium, was shown by Steinberg to be essential to growth and development of *Aspergillus niger*.

Just as the year 1939 comes to a close, another element is added to the list of elements essential to plants, namely silicon. Raleigh has just demonstrated that a variety of table beet (*Beta vulgaris*) known as the Detroit dark red, will not develop unless silicon, and that in adequate amount, be present in the culture medium. Raleigh grew his plants in culture solutions, except for the preliminary experiments, using asphalt-painted, fifteen-liter iron containers. These were sup-

plied with pin punctured, rubber tube aerators and asphalt-painted plaster-of-Paris tops. Desiring to avoid the added impurities in more concentrated solutions, the investigator grew his plants in dilute solutions of the following composition: ammonium dihydrogen phosphate, 0.0005 *M*; magnesium sulfate, 0.001 *M*; calcium nitrate, 0.002 *M*; potassium nitrate, 0.003 *M*. In four experiments described, the growing period was a little less than a month in the first two; just a month in the third experiment, and about two weeks in the fourth experiment. In this last experiment potassium silicate was specially prepared in very pure form so as to avoid any ambiguity in the interpretation of the results following from the addition of silicon to the medium. In all cases the plants made much better growth when silicon was present than when it was absent. As a matter of fact the plants receiving no silicon at all made practically no growth whatever. A curious symptom was noted in connection with silicon deficiency, namely that the leaves and the roots turned very dark. Wilting of the young seedlings was another characteristic of silicon deficiency. These results would seem to leave no room for doubt that silicon is an element essential to the beet plant and the presumption would not be too violent that it is likewise essential for other plants.

The results obtained by earlier investigators would seem to lend color to the statement just made, and the reviewer makes this pointed reference because it was only a few months ago that he himself predicted that silicon, aluminum, and chlorine would all be proved essential to plants at some time, in view of the fact that especially in the case of silicon he obtained such marked increases of growth when it was present in the medium as against when it was not. It is striking that the proof of the essential nature of silicon to the beet plant is forthcoming so close upon the heels of the prediction to which I referred. These results appear all the more significant when even with Raleigh's improved technique over that of earlier investigations, his control plants must have had a slight amount of silicon at their disposal.

On other sectors of this front, work has also been continued actively during the past year or two. While the reviewers of last year referred briefly to the demonstration by Steinberg of the essential nature of molybdenum to the fungus *Aspergillus niger*, and while we had known that the presence of molybdenum in the medium improved the growth of plants over what they would have attained in its ab-

sence, it was only during 1939 that the published work of Arnon & Stout demonstrated beyond question the essential nature of the element molybdenum to the tomato plant. Again the presumption that as a corollary to this finding, we may assume that molybdenum is essential to other plants as well as to the tomato seems justified. Further support for such a presumption is to be found in the well-known work on molybdenum in its relation to microorganisms, as represented by the investigations of Bortels, and of Burk & Horner, as well as others.

In the field of investigations with the element copper there have also been added contributions during the last year or so. Olsen (3) has emphasized the point that distilled water stored in tinned copper containers, contains a trace of copper and that in his laboratory, the distilled water was found to contain 0.6 mg. of copper per liter. When this water was used in making up solution media for *Hordeum*, *Sinapis*, and *Dianthus*, the plants seemed to thrive better and produce considerably more dry matter than the same kind of plants when grown in the same medium in which, however, the distilled water employed was obtained from a redistilled supply in a glass distilling apparatus. As a by-product of this investigation, it was found that sodium diethyldithiocarbamate is especially suitable for determining the copper content of vegetable matter. It was also found that the maize plant is perhaps more tolerant to the toxicity of copper than other plants investigated. Moreover as might have been suspected the presence of other ions in the medium may offset the toxic effect of copper. With extraordinary precautions in experimental technique, Arnon & Stout have furnished further evidence on the problem of the copper relations of plants and have developed the technique for the purification of water and salts, and for the control of contamination of the medium by containers and from other sources which constitute material assistance to further investigations.

Olsen (1) contributed further evidence to the subject of iron relations of plants. The results of his experiments emphasize the effectiveness of the pH of the medium in regard to the quantity of iron necessary to produce good growth in maize plants. These plants will grow well with comparatively small quantities of iron when the medium has a pH of 4. When, however, the medium has a pH of 7, the plants become chlorotic with the same amount of iron in the medium. Moreover, addition of larger quantities of iron to the medium when the pH is 4 may be toxic, but such procedure when the pH of the medium is 7 does not yield iron toxicity. Different compounds of

iron at a given pH vary in their toxic qualities when used in sufficiently large amounts. For example, it was not possible to show toxicity with ferric sulphate at a pH of 7 in the medium, and the greatest production of dry matter was obtained from plants when the greatest amount of ferric sulphate was added to the medium, but this was not the case when ferrous sulphate or ferric citrate was used in place of ferric sulphate. Aside from the quantitative factor and in regions of concentration which are not toxic, ferric citrate proved to be especially suitable as a source of iron for plants in culture solutions. It must not be added all at once, but at intervals during the growth of the plant. It was also found that when the mustard plant was investigated in this regard the use of ferrous sulphate (1 mg. of iron per liter of culture solution at intervals of four to five days) is necessary in addition to ferric citrate to maintain the plants in a condition of satisfactory growth.

Again in the realm of studies on the iron relations of plants, Chapman has found that citrus seedlings are capable of absorbing iron from finely ground magnetite which is mixed with sand in culture jars in which the seedlings are grown. In view of the very low solubility of magnetite in an ordinary culture solution, Chapman infers from his results that the intimate contacts between the roots of the seedlings and the magnetite particles make possible the absorption of iron by the roots from the iron oxide. This is similar to the phenomenon of contact effects between plant roots and soil colloids called to our attention by Jenny and his collaborators which is reviewed below. An interesting quantitative feature of Chapman's experiment is that with an amount of magnetite (about 0.1 per cent) sufficient to supply the seedlings with enough iron under ordinary conditions, the addition of calcium carbonate brought about iron deficiency, but with an increased amount of magnetite the plants were again able to obtain enough iron in spite of the presence of calcium carbonate. The interesting bearing of this investigation can not be detailed here because of lack of space.

When one appraises the subject of the essential elements from the historical background of the last fifteen years, one finds it difficult to reach conclusions which are consonant with the conclusions of recent investigators and at times of individual authors. It may not be fairly said in the opinion of this reviewer that the present-day investigations are more strictly quantitative and less descriptive than the earlier investigations in this field. As I see the comparison between the methods of earlier investigators and those of today, I can discern

differences in degree but no difference in kind between the earlier and the more recent attacks upon this type of problem. For example evidence will be found in the paper by Sommer & Lipman which was published thirteen years ago of the same solicitous attention to the methods employed in the investigations which in all but degree is accorded to such investigations at the present time. Emphasis was placed then as now upon the importance of chemically pure salts, of the right kind of container for the medium, of pure water and of the removal of excess storage products from the vestiges of seeds attached to the seedlings used in the investigation. In this regard the later investigations differ from the earlier ones in a number of detailed precautions and in improved methods of obtaining pure water and salts, free from contaminating substances rather than in general principle. They also differ from the earlier investigations in placing more emphasis on the criteria by which the essential nature of an element may be gauged, but in 1926 as at present it was emphasized as reference to the literature will show that one may not assume that a chemical element is not essential to plants merely because in a given investigation the results did not testify to the marked improvement in growth of plants through the inclusion of a certain element in the medium. Everything in such matters depends on technique, and it is all too obvious to any student of the subject that it is much easier to eliminate from the medium or the environment in general one element whose function it is sought to study than another. Moreover even when two elements are equally easy to eliminate from the medium one may be required by the plant in larger quantity than another and that difference alone may yield results positive for one element and negative for another. The prediction which the present reviewer made many years ago that improvement in technique of operation of such experiments would probably lead to the discovery of many more essential elements than we recognized in 1920 has been to a large degree fulfilled and it is safe to say that it will be found even more true in years to come. It is important to emphasize also the statements made recently by one or two authors in this field of our literature that less distinction should be made than is now made between the so-called major and minor elements, or macro or micro elements, or trace elements and other elements. All of these terms are adopted for the convenience of the investigator and author concerned but it is all too easy for plant physiologists and others interested to take them literally. As several of us have emphasized from 1926 forward, an element is no less es-

sential to a plant if it is at all essential just because it is used in minute quantity by the plant than another element which is used in large quantity by the plant. Obvious as this seems to me and others, it is not always appreciated by authors of our textbooks and reference books, and even at times by investigators in the field itself. No plant physiologists of today would think for a moment of according a minor position of importance to the element iron as compared to the element potassium even though iron is taken up in small quantities and potassium is taken up in large quantities, and yet I have found a tendency, and the terminology referred to above emphasizes that, to accord a minor position to copper in the list of essential elements and a major position to certain others of the well-known essential elements. In fact even in the literature on this subject published within a year, there are statements to be found to the effect that the essential nature of copper has not yet been established. There is still a strong tendency to hark back to the language of other days in which we spoke of some elements as stimulants rather than as essential elements to the life and development of the plant. It is to be devoutly hoped that that kind of distinction will make its early disappearance from the pages of our literature.

CHEMICAL ELEMENTS ESSENTIAL TO CRYPTOGAMIC PLANTS

Mention was made above of the announcement by Steinberg in 1938 that gallium is essential to the growth and reproduction of *Aspergillus niger*. Such consideration was given to this article here for two reasons: In the first place the burden of evidence obtained in such studies to date indicate that they do not prove that the elements which are essential to higher plants are also essential to lower plants and vice versa. Steinberg's discovery, therefore, should form the basis of studies seeking to determine whether or not gallium is essential to the life of higher plants. The second reason for directing attention to this contribution is that we are in dire need of many more investigations on the requirements of the chemical elements by microscopic plants. It is undoubtedly owing to the fact that the technique for working on such a subject requires much more refinement even than that accorded to the most difficult studies with the essential elements for higher plants. This, however, should not prove a deterrent to investigations to seek more definite information in regard to these phases of the inorganic metabolism of algae, fungi, and bacteria, and

particularly to such classes of organisms. The reviewer expresses the earnest hope that such investigations will soon enlist the interest and activity of investigators in this field to a greater degree than heretofore.

FUNCTIONAL ASPECTS OF THE ESSENTIAL ELEMENTS

In describing some of his own investigations in the search for additional essential elements, the writer has several times pointed out that the most difficult and most important of all problems in connection with the essential elements is the one which involves the function or role played by each of these elements. As I review the subject today I find myself obliged to call attention to the fact that while this problem remains as important as ever, we have made relatively little progress towards its solution. It is one thing to prove beyond a doubt as investigators have already done that boron is essential to plants. It is quite another and far more difficult and elusive problem to prove why boron is essential to the plant. I do not desire in the slightest degree to detract from the credit which most properly belongs to the investigators who have in various ways contributed to our present knowledge of the identity of the essential chemical elements, but in all candor it must be stated that these praiseworthy contributions have thrown little or no light on the functions of the several chemical elements which are essential to the plant. With equal candor I am more than ready to state that attempts have not been lacking, however, to attack these extremely difficult biochemical and physiological problems in recent years. It seems appropriate to review a few of the contributions which might be said more or less properly to belong to this field even though they may not in any or all cases approach the heart of the problem.

Russell has shown that when barley plants are grown with the element potassium deficient, there is a notable decrease in the fructosan content of the barley tissue and that this is especially true when the sodium content of the medium is high. He has shown further that when barley plants are grown in a medium deficient in phosphorus, the fructosan content of the plant is increased. The results indicate further that the ratio of fructosan to other sugars in the plant is similarly affected as to its absolute content by the deficiencies of potassium and phosphorus in the medium. No explanation of why the elements of potassium and phosphorus function in this way is offered.

An article of a similar order has come from the pen of Day & Comboni and an additional one from Comboni alone. In the first article we find a report of experimentation on the influence of potassium deficiency on the formation of starch in *Pisum*. Plants were grown from seed in sand cultures with complete nutrient solution and other plants with similar solutions in which potassium phosphate was replaced by ammonium phosphate. Qualitative and quantitative tests were made on the leaves for starch. The qualitative tests were made with iodine and the quantitative tests by the method of Pucher & Vickery. The plants which did not receive potassium showed necrosis of leaves first in the older leaves and then in the younger and also produced fewer leaves and smaller ones than the control plants. On the basis of dry weight there was always more starch found in the higher leaves; the higher up on the stem the more starch. The leaves on the control plants always had the most starch. The plants receiving half as much potassium as the control plants had about three-fourths as much starch as the controls. The plants receiving no potassium had less than half the normal amount of starch. The article by Comboni gives an account of anatomical observations on the seedlings of *Pisum sativum* grown under similar conditions to those described above. In connection with the last named article the reader is referred to another paper by Wall which bears on a similar subject.

On the subject of calcium-magnesium relationships in plants we may now review certain investigations which seem of interest. Sambo reports an investigation of the lichen, *Diploschistes ocellatus*, found on rocks in the Mt. Ferrato district in Italy. This lichen forms white thalli 10 to 50 cm. in diameter on granite rock and under those conditions produces large apothecia. He found this lichen growing also, however, on serpentine where only a trace of calcium is present but which contains 36 per cent magnesium. The lichens on the serpentine are grayish in color, only about 1 mm. in diameter and with correspondingly small apothecia. These lichens on serpentine become white on treatment with calcium hypochlorite and greenish brown with potassium hydroxide. The ordinary form of the species on granite shows no such reaction. Sambo claims that various other calcicolous lichens are able to grow on calcium free media if magnesium is present. These observations have an interest in this review because they indicate the power of magnesium to take the place of calcium for this type of plant. They do not, however, prove as some have assumed in this and other cases that the lichen does not require

calcium. Somewhat similar in nature to this contribution by Sambo is the one by Trelease & Selsam. *Chlorella* was grown in media containing calcium chloride and in similar media without calcium chloride. The amount of growth made by *Chlorella* in the medium containing calcium chloride was no greater than that in the medium in which calcium chloride was not included. It was noted, however, that in the absence of calcium, *Chlorella* tolerated high concentrations of magnesium salts and it even attained considerable growth in a solution which contained 0.42 *M* magnesium sulphate. When magnesium was added to the point of toxicity, additions of calcium did not diminish the toxicity of magnesium. Conclusions from these observations should be drawn with great caution as indicated also by Sambo's investigation.

Mullison has contributed to our knowledge of the effect of the deficiency of calcium on the respiration of etiolated seedlings. The total respiration of plants, of pea and corn grown in culture solutions lacking in calcium was in every case less than that of plants furnished with complete solutions. The effect of the deficiency of calcium was most noticeable in the greatly lowered respiration of the tops of the plants grown without calcium as compared with that of the plants grown in complete solutions. This added evidence on functional relations of calcium to respiration is doubtless important to us.

Among other contributions, which have been made during the past year or more, to our knowledge of the functional activity of some of the essential elements, the following are in the reviewer's opinion worthy of special attention. Bertrand & Silberstein have shown that the boron content of the lily plant including the flowers is comparable to that of the onion or garlic plant, namely about 4 mg. per kg. of dry matter. The greatest concentration of boron is found in the stigma which is shown to contain as much as 12.8 to 14.3 mg. of boron per kg. of dry matter, as against 2.5 to 3 mg. in the stalk, 4.25 to 5 mg. in the roots and bulb, and 9.6 to 10.9 mg. in the leaves. This points to an unusually intense utilization of boron by the reproductive organs which has been hinted in articles which have appeared earlier. In a similar investigation Bobko & Tserling have arrived at similar conclusions in regard to the boron content in stigmas of flowers and in pollen.

Another contribution looking toward the elucidation of the function of potassium is offered by Pirson. This deals with the possible relations of potassium to photosynthesis. Pirson believes that when

Chlorella is deprived of potassium in the medium and is later supplied with that element, photosynthesis revives in two phases: first the potassium ion acts directly upon the weakened mechanism; secondly, improvement in the photosynthetic process itself takes place which Pirson connects with the new formation of chlorophyll and other cell materials. In the first phase rubidium can take the place of potassium, but cesium can only partially do so. Cesium does not maintain mitosis nor does it permit of the increase of chlorophyll. He points out further that respiration is independent of photosynthesis in these respects and potassium, rubidium, and cesium all interfere with the increased respiration of cells which are starved for want of potassium. In the presence of potassium, cesium, and in a higher degree rubidium, is beneficial to *Chlorella*. These observations and facts are somewhat confusing to one who seeks to find simple functional relationships between the essential elements and life phenomena in plant cells. It is highly probable, however, that simple functional relationships can not be expected in such complex systems as those which constitute cells.

Still another aspect of the functional activities of the essential elements is treated in a contribution from Penston. The leaves of maize plants were studied at hourly intervals, using leaves of three to four, five to six, and seven weeks of age, and the content of dry matter, ash, water, and potassium, was determined at these intervals also. It was noted that all of these constituents increased in the leaves in the morning, fell to a minimum at about 11:00 a.m. and rose again in the afternoon. The magnitude of these changes increased with age to the point of maturity, but then it no longer increases in proportion to the basic weight of the leaves. The author concludes from these observations that absorption and movement of mineral elements in leaves during the day evidently depends on the rate of metabolic activity. Interesting as these observations are, it seems to the reviewer questionable as to whether they render clearer or more obscure the complicated relationships of say potassium alone to the functioning of the cells.

An interesting addition to our knowledge of the mineral metabolism in plants is furnished by Ketchum who worked with unicellular plants. When *Nitzschia closterium* is grown in a medium deficient in phosphorus in the light, it forms cells which are deficient in phosphorus. When phosphorus is added to the medium, the cells absorb it rapidly even when they are in the dark. While cell division does

not take place in the dark, the phosphorus absorption continues and is completed in ten hours. These experiments show further that the amount of phosphorus absorbed is independent of the concentration of phosphorus in the medium, but is directly related to the length of time during which the cells are grown in a phosphorus-deficient medium in the light. The amount of phosphorus absorbed per cell in the dark is a direct measure of the deficiency to which the cells had been subjected and is called the phosphate or phosphorus debt. The author infers that precursors of organic compounds of phosphorus can be formed in the light in the absence of phosphorus in the medium. In illuminated cultures an equal amount of phosphorus is absorbed for every cell formed. The author believes further that the phosphorus which is absorbed in the light also combines with the same constituent of the cell and claims that in similar experiments with *Chlorella pyrenoidosa*, similar results were obtained as with *Nitzschia*. This alga absorbed in darkness as well as in the light nitrogen as well as phosphorus to make up deficiencies suffered by them when grown in nitrogen and phosphorus-deficient media. The reviewer does not consider it necessary to make any further comments on this report by Ketchum since it speaks for itself.

An important observation was made by Postma during the past year. This concerned the conditions under which nitrate reduction affects respiration of roots. He grew oat seedlings in Knop's solution minus nitrogen for thirteen days in the light, and then placed the plants in darkness for two days. The stem and leaves were then removed and the roots were kept two more days in the dark in a solution containing nitrate or glucose, a solution which contained neither, and a solution which contained both. Determinations of total nitrogen and protein in the roots following this experiment showed that both total nitrogen and protein decreased in the following order: nitrate plus glucose, glucose, nitrate, and a solution without either. The carbon dioxide production by the roots in the solution just noted was as follows per twenty-five plants: 0.36; 0.057; 0.057; 0.037 mM in the solutions just named and in the same order. In distilled water the value was 0.029 mM. The author concludes with perfect justification that with glucose present, nitrate is readily reduced, protein is formed, and respiration is abundant. These results serve to confirm and amplify earlier results on the role of roots in nitrate reduction.

A final item of interest in this category is the report by Trelease

& Trelease on the "Physiological differentiation in *Astragalus* with reference to selenium." These authors have found that *Astragalus racemosus* grown in solution and sand cultures was much improved in its growth by the element selenium in concentrations from 0.33 to 9 p.p.m. Another species, *Astragalus crassicaarpus*, was not stimulated by any concentration of selenium, but on the contrary was severely injured by a concentration as low as 0.33 p.p.m. This investigation is mentioned, therefore, primarily because of the new aspect which it gives to the problems of inorganic metabolism of the higher plants. Two species within the same genus show physiological differentiation in their responses to one and the same chemical element. Therefore, the question as to whether or not selenium is essential to *Astragalus* is complicated by a species differentiation of a physiological order. It is, of course, obvious that selenium may be essential to both species and yet one of them may be poisoned by a concentration of selenium which merely stimulates another one. The species which showed the higher tolerance for selenium was able to accumulate correspondingly higher concentrations of that element from culture solutions.

ABSORPTION AND ACCUMULATION OF SALTS

With the exception of one article which is discussed below, the reviewer has not noted contributions in the literature on the subject of absorption of salts and accumulation of ions during the past year which alter in any appreciable way the facts and principles which prevail in that field today, or which prevailed in that field a year ago. A great many contributions to the fundamental subject of permeability have been made during the past year and during the latter part of 1938 in the investigations of Osterhout, Jacques and their collaborators. Further contributions have also been made by Brooks on this subject, but the reviewer does not find in these papers anything which requires particular comment in this review. An important note on the general subject of metabolism and salt absorption by plants deserves special mention in connection with the foregoing comments on the field as a whole. This note was published by Hoagland & Steward and gives a clear and critical examination of Lundegårdh's attempt at a physical theory of salt absorption by plants. When one reads carefully this critical review of the theory by Hoagland & Steward, one cannot help but feel that they take a sound position respecting the Lundegårdh hypothesis. After all a complex system of root cells with its complicated connections all deeply affected by the environment

of the roots as well as the tops of the plants cannot lend themselves to any very simple explanation of the phenomena for which they are responsible. All those who have studied ion absorption by cells and the various factors which influence ion absorption in plants must agree that Lundegårdh's views on the role of anions are nothing less than an overemphasis on one aspect of the whole complex subject of ion absorption. In any case everyone interested in the subject of metabolism and salt absorption by plants owes a debt of gratitude to Hoagland & Steward for their scholarly review of the Lundegårdh hypothesis.

Of the original contributions in this field I wish to mention the paper by Steward & Harrison which it seems to me does more than any other paper which I have studied in this field to give us something new to think about in relation to the general subject of the absorption and accumulation of salts by plant cells with special emphasis on the uptake of rubidium and bromine. With the help of special spectrographic technique and with additional apparatus specially devised for their purpose, Steward & Harrison have carried out experiments to obtain facts on the absorption of rubidium and bromine from rubidium bromide. They employed their usual methods of control for aeration, temperature, effective stirring, etc. Potato discs were used as the absorbing material and the absorption of rubidium bromide was studied in connection with oxygen supply, time, and the specific surface of the discs. The result leads the authors to the following extremely interesting conclusions: The absorption of rubidium from rubidium bromide is of two kinds: the first is independent of the oxygen supply, and in this case rubidium enters rapidly but not the bromide. Moreover this process is not limited to the surface cells but to the whole mass of cells in the disc and ceases after a short time. In the second phase of this absorption process we have a prolonged period in which rubidium and bromine are both absorbed and in equivalent amounts. This process is definitely influenced for both ions, by the oxygen supply, and the absorption is limited to a few layers of cells. The authors ascribe the first of the two phenomena to the properties of the substances in the tissue and call it induced absorption; whereas they regard the second of the two phenomena as a property of the organized cell which demands that work be done to accomplish it and this they refer to as "primary absorption." If these facts and explanations are found to be unassailable, we have at our disposal a satisfactory explanation of the unequal uptake of rubidium

and bromine from rubidium bromide and this becomes a very distinct and important contribution to our knowledge in this field.

A number of other investigations are selected by the reviewer for comment and classified under this general field of salt absorption just for the sake of convenience. The first is the investigation by Hurd-Karrer and deals with a certain phase of the subject of antagonism between ions with special reference to the antagonism between essential elements and so-called nonessential elements, chemically related to them but which are referred to as toxic. Hurd-Karrer bases her views on experiments which led her to the conclusion that when varying degrees of antagonism are shown between ions, as illustrated in her data, they result from the merely partial selective power of the plant, or in other words the plant is in some degree unselective as regards the absorption and utilization of chemically related elements. She cites the following examples in support of her contention. Arsenic injury may be regarded as a function of the available phosphorus concentration in the medium, with the protective As/P ratio in the medium near 1:5. Similarly the toxicity of rubidium is a function of the available potassium concentration with the protective ratio Rb/K near 1:2. Strontium injury is a function of the available calcium concentration with the protective Sr/Ca near 1:1. The assumption is made further that the chance of substitution with a harmful effect of a toxic for a useful element when an organic molecule is synthesized depends on the proportion of the availability of the two. The author calls this mass absorption. It would seem to the reviewer that these explanations must be accepted with caution. Those who have carried on investigations in the field of antagonism between ions have, I think, had the common experience of noting how markedly changes in the composition of the medium affect antagonism phenomena as well as absorption of salts regarded from the absolute standpoint. The reviewer's own experience in this field leads him to be very cautious about the acceptance of ratios between the antagonizing elements as reliable guides.

Olsen (2) in an investigation of the absorption of calcium and the formation of oxalic acid in higher green plants has found among other things that the amount of calcium which is not in the form of calcium oxalate in certain species of plants, for example, *Picea excelsa*, is independent of whether a greater or smaller amount of calcium is absorbed. In other species the amount of oxalate calcium increases when the amount of absorbed calcium exceeds a certain

limit. "In these species the oxalic acid produced cannot quite keep up with the absorption of calcium when the plant is growing in a calcareous soil from which it absorbs large amounts of calcium." Other observations made by Olsen are interesting. In the oxalic acid producing species, *Fagus silvatica*, 62 per cent of the total calcium content of the leaves was found in the form of oxalate and 9 per cent was found in the cell sap in dissolved form; whereas in *Tussilago farfarus*, which does not form oxalic acid, only 35 per cent of the calcium content of the leaves was present in the cell sap in dissolved form. This investigation carries with it some further evidence on the antagonism between ions. It was found that the ammonium ion inhibits calcium absorption by the plant to a greater degree than does the potassium ion. Finally experiments with *Dianthus barbatus* showed that it may make good growth in the presence of only small quantities of calcium; for example when cultivated in a solution containing only 4 mg. of calcium per l. with ammonium sulphate as a source of nitrogen, it showed a calcium content of the leaves amounting to only 0.1 mg. of calcium per gm. of dry material. This, in the reviewer's opinion, should not be regarded as proof that the *Dianthus* plant can get along without calcium. Moreover, I judge that Olsen agrees with this view.

One of the important investigations of the year which the reviewer has elected to place in this category is that by Stout & Hoagland on the upward and lateral movement of salts in certain plants. This investigation not only offers important objective evidence on salt absorption as such but also makes a contribution of first importance to our understanding of the path followed by salts in their upward and lateral movements in vascular plants. By the use of radioactive isotopes of potassium, sodium, and phosphorus and by the separation of the bark from the wood in some sections of the stem, it was possible to prove through the use of willow and geranium plants that the main if not the entire amount of salts moves upward in the wood and it is only owing to the contact of the bark with the wood that salts are transmitted laterally to the bark. This evidence then supports strongly the prevalent hypothesis of plant physiologists and plant anatomists that the xylem and not the phloem "is the path of rapid upward movement of salts."

Finally mention should be made in this category of two investigations, one by Reed and another by Reed & Beck, which deal with the influences in the first case of copper and zinc on leaf structure and

the other case with the effect of zinc on the growth process. In the first investigation detailed anatomical evidence is given of the pathological changes taking place in tomato plants when copper and zinc are deficient in the medium; whereas in the second study in which maize was used, the following effects on the growth process were noted when zinc was deficient in the medium. There was a curtailment of cob and kernel production more than in stalk, leaves and husks. The medium used was the Delhi soil with and without the addition of zinc. The grand period of growth of the plants grown with zinc deficiency and with an adequate supply of zinc was the same.

THE ABSORPTION OF IONS BY ROOT CELLS THROUGH DIRECT CONTACT WITH COLLOIDS

The reviewer has elected to place in a separate category the type of absorption indicated in the foregoing title because of the striking nature of the investigations involved which set them apart, as it were, from the general phenomena of absorption discussed above. Jenny and his collaborators have continued their researches on contact depletion of barley roots and related phenomena. In one investigation the study of contact depletion was made with radioactive indicators, and it was found among other things that barley plants retain radioactive potassium against distilled water and release it to salt solutions and clay suspensions. "For equal amounts of cations in the nutrient media, the colloids greatly exceed the single salt solutions in removing the radioactive potassium from plant roots. The outgo of radioactive potassium from plants increases with concentration of nutrient media and time of contact."

Similar results were obtained in working with radioactive sodium which show again that colloidal clay suspensions are far more effective as depleting agents than salt solutions of corresponding cation concentrations. On the other hand it was found that in the case of plants containing radioactive bromine, the clay suspensions removed similar amounts of the isotopes from the roots as do the salt solutions. Most important of all, this investigation confirms earlier observations by the same investigators to the effect that the intake of ions by the roots is not a unidirectional process but ions of the same species may move into the root and out of the root at the same time.

In the reviewer's opinion the importance of these investigations

cannot be overemphasized since they modify in a fundamental way our conceptions relative to the source of important ions to plants as well as to the mechanism by which absorption is accomplished, at least in terrestrial media. While these investigations render the processes in question more complicated than was postulated on the soil solution theory alone, they help to explain many observations which for more than a generation have proved confusing and perplexing to the investigators interested in the problem of soil and plant interrelations.

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SOIL MICROBIOLOGY¹

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The nature of the soil population.—The methods used in the study of the microbiological population of the soil continue to receive considerable attention. In proposing the method of direct staining of soil, Conn emphasized that the study of soil bacteria in pure cultures led to confusion, because "pure cultures never occur in the soil." Winogradsky (1) went a step further and insisted that "soil microbiology is not mere bacterial physiology, but has to deal with the ecology of the microbial soil population." This population was analyzed in accordance with the functions of the organisms in the soil; their specialization in the soil, due to natural competition, was determined by means of enrichment culture methods. Winogradsky emphasized that a distinction should be made between the autochthonous or native population of the soil, and the zymogenous population, which develops only when the soil is enriched with fresh nutrients. The contact or overgrow slide technique (2), designated by Rossi (3) as the soil impression method, was utilized for the study of the microbiological state of the soil; for the growth of pure cultures of bacteria in soil and abundance of specific groups of bacteria; for a study of the microbial response to fertilizer treatment and changes in soil condition; for development of specific organisms in response to additions of organic compounds and changes in the specific nature of the population; and to study parasitism of certain organisms upon root systems of plants, and relationships between the saprophytic soil microorganism and plant roots. Bacteria were found (3, 4) to be represented in the soil both as isolated cells and as aggregates, which were designated as "cysts," "zoogloea," "clusters," and "glomerules," the small, nearly spherical bacteria being the most abundant constituents of these. The clusters of bacteria were believed to be characteristic of aged soils indicating a state of repose, similar to protozoan cysts; upon cultivation, these clusters burst open and the cells become active; large, *Azotobacter*-like cells, sporeforming bacteria,

¹ An attempt was made to cover, in this review, the available literature up to September 10, 1939.

and long, slender spindles of the *Cytophaga* type were less frequently encountered.

Actinomycetes were found in great abundance in the soil, both in the mycelial stage and in the form of spores (4, 5); due to the ease with which these organisms are stained, their distribution in soils and in composts (6) can be easily brought out by the contact slide method. The abundance of fungus mycelium found by this method emphasizes again the extensive growth of fungi in soils, where they form an important constituent part of the population. Among the other microorganisms detected by the contact method, the algae, protozoa, nematodes, and insects received attention; these organisms are not uniformly distributed through the soil mass but are localized on organic matter particles (4, 7) or upon the debris of larger organisms, such as plant roots, insects, and fungus mycelium.

Some plants and even soils contain substances which are microbicidal in nature (8), whereas others, notably leguminous plants, produce substances favorable to many or to certain specific groups of microorganisms. In general, the distribution of microorganisms in relation to plant development is directly dependent upon the production by the plant of new epidermal and other cells, and the root hairs at the growing tip, all of which die off, after a short functioning period (4, 9). The formation of specific plant-stimulating substances of a hormone-like nature by microorganisms is still a matter of dispute (10). The "soil chamber" and "soil dust" methods proposed by Cholodny (2) for studying the development of the soil population in an undisturbed condition were found to be applicable for fungi (11) as well as to a more limited extent for bacteria and actinomycetes.

The plate and dilution methods, however, are still used extensively for determining the relative abundance of soil microorganisms. The addition of growth-promoting substances to the culture media, in order to avoid the variability of plate counts, was suggested (12); the medium is therefore no longer synthetic. Attempts were made to establish mathematical relations between the abundance of microorganisms and certain specific activities, on the one hand, and soil moisture and temperature, on the other (13). Different soil types were found (14) to possess characteristic microbiological populations, as determined by these methods; relations between microbial activities and crop yields were established only for certain soils, although a definite correlation seemed to exist between optima for plant and microbial development. A study of the distribution and activities

of bacteria in the different soil horizons revealed (15) the greatest numbers and activity in the horizons high in organic matter, namely the A₁, followed by the B₂; marked seasonal changes were also observed. In their physical relation to the soil, the microorganisms were found (16) chiefly upon the solid particles; only a small percentage was found in the liquid phase. This is due to the adsorption of the organisms by the soil, the intensity of adsorption being determined by the mechanical composition of the soil, nature of the organisms, and soil reaction. The base predominating in the exchange complex is of special importance in this connection, monovalent cations giving the least adsorption. The evolution of carbon dioxide by adsorbed bacteria was retarded in proportion to the adsorption capacity of the soil. In other experiments, however, the adsorption of bacteria, most pronounced in the cases of pure cultures, had no paralyzing effect upon their activities (17).

The microbiological population of the soil was shown to undergo marked fluctuations through the year (18). The short term variations arise from competitive factors whereas the long term changes are a reflection of seasonal changes in climatic conditions as affecting the supply of energy for microorganisms provided by plant materials. This explanation was offered as being more logical than the one suggested previously by Cutler & Crump and based on an "inherent urge." The abundance and distribution of microorganisms in soil as well as the specific nature of the population of different soil types was found to be influenced differently by additions of organic matter (19).

Filterable forms and lytic agents.—The growing interest in the nature of viruses, phages, and other filterable agents is reflected in certain studies of soil organisms and their relation to plant and animal parasites. These agents can be classified in three distinct groups: Group I comprises definite living systems which have been defined as filterable saprophytic organisms (20), capable of cultivation on artificial media. Group II includes products of living systems which are more related to viruses and phages, some of which have enzymatic properties (21); the specific phages found in soils comprise not only bacteriophages, but also actinophages and mycophages (22); the role of certain specific phages in injuring the growth of leguminous plants is of particular importance (23); some phages may be beneficial by destroying plant pathogens, such as *Bact. citri* (24). Group III consists of well-defined chemical products of soil microorganisms which have a destructive effect upon other organisms; of particular interest,

in this connection, are the recent findings of Dubos (25) that the soil contains bacteria exerting a bactericidal effect on gram-positive organisms, but having no such effect on gram-negative species. The specific spore-bearing soil bacterium which was isolated, produced an active substance which is alcohol- and acetone-soluble, nonvolatile, heat-labile, and nondialyzable; it is not a protein, although it contains 12.5 per cent nitrogen.

The specific lytic effect of various soil bacteria against fungi may also be mentioned (26); this phenomenon has a bearing upon the common observation that the growth of fungi in a mixed soil population is usually followed by extensive development of bacteria. Certain myxobacteria were isolated from the soil by the use of living fungus mycelium as a substrate.

Autotrophic bacteria.—The autotrophic nature of the classical ammonia-oxidizing organisms (*Nitrosomonas*) has again been emphasized; soil extracts and casein digests were shown to have a favorable effect upon the growth of these organisms on agar media (27). The common observation that nonnitrifying organisms may exert a stimulating action upon nitrifiers was explained as due to a phenomenon of symbiosis (28), a point which can still be questioned.

The method of isolation of nitrifying bacteria was facilitated by the use of silica gel media and by plating a suspension rich in the specific organisms (29). It has been reported (30) that nitrate may be formed in composts kept at a temperature of 75° C.; the nature of this process is still unknown. Recent claims that nitrate formation in soil is predominantly photochemical or physicochemical in nature (31) were not substantiated by further studies (32); the same is true of the claims concerning the oxidation of ammonia by the protozoan *Chilomonas* (33). The oxidation-reduction potentials in soil were found (34) to have important effects upon the formation or reduction of nitrate. Boron is favorable to nitrification; this element was shown to be fixed in the soil in the process of decomposition of plant materials by microorganisms, thus removing this element from circulation (35).

The autotrophic nature of the organisms responsible for the precipitation of manganese in certain soils, which are believed to be responsible for the manganese deficiency in oats (36), is still open to question. The same is true of the bacteria which are said to activate molecular hydrogen, with sulfur as the acceptor, according to the reaction: $H_2 + S = H_2S$ (37).

Nitrogen-fixing bacteria.—Certain cultures of *Azotobacter* were found in Indian and in Malayan soils capable of growing at wide reaction ranges, pH 3.6 or even 3.0 being the limit on the acid side (38). The abundance of *Azotobacter* in soil is influenced by fertilizer treatment (39) and by a number of special elements, such as molybdenum, vanadium, and tungsten; these have a marked stimulating effect upon the growth and nitrogen fixation of this organism (40). The effect of molybdenum takes place only in the presence of iron; both of these elements can replace humus in its effect upon nitrogen fixation (41). The mechanism of the process of nitrogen fixation is closely associated with the life activities of the cell (42); the existence of specific enzyme systems, upon which much speculation has been recently based, has so far not been established experimentally.

Phenol and benzoic acid can be used as sources of energy for nitrogen fixation by *Azotobacter*, 9 to 11 mg. nitrogen being fixed per 1 gm. of phenol consumed in an elective medium containing 0.05 per cent phenol (43). High concentrations of combined available nitrogen rendered *Azotobacter* incapable of fixing atmospheric nitrogen; in some, but not in all strains, this property was restored by growing the organisms in the presence of only very small amounts of combined nitrogen (44). The fixation of nitrogen by *Azotobacter* was found to be injuriously affected by pigments produced by certain organisms, such as actinomycetes (45).

The ideas of Löhnis concerning the formation by *Azotobacter* of filterable stages and gonidia were not substantiated; the organism was found to give abnormal growths (adaptates and mutilates) in response to special treatments (42). *Azotobact. agilis* and *Azotobact. vinelandii* were shown to be distinctly different organisms, the latter being a characteristic inhabitant of soils and the former of canal waters (46). *Azomonas* was suggested (47) as the name for the non-cystogenic organisms commonly found in waters. In refuting Löhnis' pleomorphic conceptions, Winogradsky emphasized the need for using, in investigations of this type, organisms freshly isolated from natural substrates and for cultivating them upon media containing alcohols instead of carbohydrates; pathological morphology was thus differentiated from normal morphology. No symbiosis could be detected between cellulose-decomposing and nitrogen-fixing bacteria (48). Many anaerobic saccharolytic bacilli were found (49) to be capable of fixing nitrogen; *Clostridium pastorianum* was most active. Under special conditions of growth, *Clostridia* were able to use some of the

intermediate substances produced by fungi in the decomposition of cellulose (50).

The association of root nodule bacteria with leguminous plants continues to occupy the attention of many workers (51). The plants exert a chemotactic effect upon the bacteria, which congregate around the plant roots; the bacteria secrete a substance (auxin) which causes the curling of the root hairs of the plant (52). The bacteria, acting as parasites during the early stages of growth of the seedling, may enter the host through the root hairs or through ordinary epidermal cells (53). The presence of nitrate represses the formation of nodules; the inefficiency of certain strains of bacteria seems to be due to induced production by the host of a substance harmful to the bacteria, inhibiting their growth and causing their disintegration (54).

Barium adsorbed on a clay colloid has been reported to be responsible for the development of variant types of cultures, whereas titanium increased nodulation and nitrogen fixation (55); the nature of the exchangeable bases in soil and degree of saturation are highly important in the development of at least certain soil microorganisms (56). Certain claims have been presented concerning nitrogen fixation by the legume bacteria, in the absence of the host plant, but in the presence of "bios" (57); these claims have not been confirmed (58). It has also been reported that excised nodules are capable of fixing nitrogen in the presence of oxalacetic acid (59); this claim as well has so far not been substantiated. The earlier claims of Vita concerning the fixation of nitrogen by germinating peas were found (60) to be due to errors inherent in the Kjeldahl method of analysis.

The question of growth-promoting substances required by *Rhizobia* has attracted considerable attention (61). These substances have frequently been designated as "Coenzyme R," and were found to consist of vitamin B₁ and other accessory food substances; they influence both growth and respiration of the *Rhizobia*. There seems to be some controversy as to whether they operate by means of modifying the oxidation-reduction potential or whether they are specific for growth.

Winogradsky (62) presented evidence to show that ammonia is one of the first products of nitrogen fixation by root nodule bacteria; the fixation process was considered to be enzymatic in nature. A detailed study of the comparative nitrogen metabolism of plants actively fixing nitrogen or fed combined nitrogen, as well as a detailed analysis of the nitrogen components in the nodules showed that only

arginine-like compounds could be directly attributed to the nitrogen fixation process (63). The compounds found in the substrate after the growth of inoculated legumes were shown to be *l*-aspartic acid and its decarboxylation product β -alanine (59, 64); this has served as a basis for a proposed mechanism of nitrogen fixation, involving the formation of hydroxylamine from atmospheric nitrogen; the subsequent combination of the former with oxalacetic acid to form *l*-aspartic acid through the oximinosuccinic acid stage. This mechanism is still questioned (63, 65), especially in view of the fact that *Azotobacter* also produces oximes in cultures containing nitrate nitrogen; the oximes are later changed to imino and finally amino groups; this process does not involve the formation of hydroxylamine (66).

Nitrogen fixation in red clover is independent of the partial pressure of nitrogen of the atmosphere until the latter is reduced to 0.1 atm., at which level fixation decreases with further changes in nitrogen pressure. The partial pressure of oxygen has no influence either on nitrogen fixation or on the uptake of combined nitrogen between 0.1 and 0.4 atm., and since oxygen pressures above or below these values decrease the uptake of free or combined nitrogen in the same degree, it was concluded that oxygen *per se* was not directly concerned in the fixation process. Hydrogen was shown to inhibit the fixation of nitrogen but to have no effect upon the uptake of combined nitrogen (67). Since the inhibition was proportional to the pressure of hydrogen and since no similar effects have been found with *Azotobacter*, this may point to a fundamental difference between the two nitrogen fixing systems (68).

Studies of the cross-inoculation groups have been continued. It has been claimed (69) that not enough plant species or diverse strains of organisms have ever been employed to justify well-defined plant-bacteria groups; some plants will interact with several strains of *Rhizobia*, whereas other plants are restricted as to the number of bacterial strains; those plants which are cross-pollinating are inoculated by more bacterial strains than the self-pollinating plants. The environment of the plant, intensity of light, and presence of combined nitrogen were all found to influence the ability of a strain to invade a certain plant (70). Marked variation was observed (71) in the quantity of nitrogen fixed when a single strain was used to inoculate different plants belonging to the same cross-inoculation group. The balance between carbohydrate and nitrogen in the host plant exerts a certain regulatory effect on symbiotic nitrogen fixation (72). This balance is receiving attention as a factor in nodule production, in the

ability to fix nitrogen, as the fundamental cause for the inhibiting action of high light intensity and in the prolongation of the "nitrogen hunger" period.

The associated growth of legumes and nonlegumes has received a great deal of attention; excretion of nitrogenous compounds by the legume has been shown by a considerable amount of experimental work (73). Excretion seems to depend on a number of factors, some of which are not yet understood (74). In the symbiosis of bacteria with other plants, such as *Ardisia crispa*, the significance of the bacteria was ascribed to the secretion of a growth-promoting substance (75).

Cellulose-decomposing bacteria.—The decomposition of cellulose and related compounds in soils and in composts is brought about by a great variety of organisms belonging to the bacteria, fungi, and invertebrate animals. Among these, the bacteria continue to attract considerable attention. These comprise aerobic and anaerobic organisms. The division of the aerobic bacteria into three groups, as suggested by Winogradsky, is now commonly accepted. These groups comprise: (a) *Cytophaga*, long, slender, flexible organisms, either forming or not forming a sporoid or microcyst; (b) *Cellvibrio*, including various vibrio-shaped bacteria; (c) *Cellfalcicula*, spindle-shaped, motile rods; other rod-shaped bacteria, sporeforming and nonsporeforming, may also be included in this group. The cyst-forming *Cytophaga* was found (76) to be closely related to the myxobacteria, whereas the noncyst-forming types are considered as transition forms between myxobacteria and true bacteria. The decomposition of cellulose by these bacteria is controlled by the amount of available nitrogen (77). The production of sugar as an intermediary product of cellulose decomposition has been demonstrated (78).

Among the anaerobic cellulose-decomposing bacteria those that occur in the digestive tract of ruminants (*Plectridium cellulolyticum*) and of termites deserve consideration (79). Methane was found to be produced by anaerobic bacteria only in very small amounts, especially in unheated cultures; its production was ascribed to accompanying bacterial impurities (80).

Sulfate-reducing bacteria.—The specific vibrio which reduces sulfate to sulfide and which is widely distributed in soils is quite unique in that it is the only vibrio which has been shown to produce spores (81). This organism was found to be concerned in the anaerobic corrosion of iron and was believed to be responsible for considerable destruction of iron pipes buried in water-laden soils (82).

Nonspecific bacteria.—The indigenous microflora of the soil is

commonly believed to be unstable physiologically and to possess considerable adaptability; it was suggested that the activity of different species is greater in associations (83). This may be one of the reasons for the confusion still existing in the morphological and functional classifications of the heterotrophic bacteria in the soil. The rod-shaped nonsporeforming soil bacteria have been classified (84) into four groups: (1) Gram-positive, motile, capable of producing branching variants; (2a) Gram-positive, nonmotile, rod-shaped bacteria; (2b) Gram-positive, nonmotile bacteria, producing filaments; (3) Gram-negative bacteria, motile and nonmotile. *Corynebacteria* were largely found in group 2a; the *Proactinomyces* were placed in group 2b.

Soil algae, fungi, and actinomycetes.—Algae have been reported to occur abundantly in various soils, including desert soils (85). Among the activities of soil algae, the most interesting fact to be brought out in this connection is the ability of some of the blue-green forms (*Nostoc*, *Anabaena*) to fix atmospheric nitrogen (86). The ability of algae to bring about losses of nitrogen from nitrate, as a result of interaction of the nitrite produced by reduction of the nitrate with amino acids, is of special interest in interpreting the losses of soil nitrogen (87).

Our knowledge of the fungus population of the soil and its role in soil processes is increasing rapidly. A detailed summary (88) of the occurrence of fungi in the soil established the fact that these organisms form an important constituent part of the soil population, extensive populations of fungi being reported from Indian, Egyptian, British, and Russian soils. The *Myxomycetes* are capable of ingesting large numbers of bacteria, thus pointing to their possible role in controlling the bacterial population of the soil (89). Some fungi were found to be capable of attacking nematodes (90). A direct correlation was found (91) between soil fertility and abundance of fungi, although soil reaction, abundance of organic matter, and nature of crop grown were also of importance. Frequently, certain specific soil fungi, as *Trichoderma kōningi*, were taken as indices of fungus distribution (92).

Soil fungi are found on many seeds and fruits; they have a marked influence upon the germination and development of horticultural plants (93). The occurrence of fungi in the digestive tract of termites has been demonstrated, although their role in the digestion of cellulose still remains to be elucidated (94). Garrett (95), in reviewing the literature on the ecology of root-infecting fungi, recognized a difference between soil inhabitants and soil invaders. The former are

primitive or unspecialized parasites of a wide host range and are widely distributed in the soil; the second group comprises specialized parasites, their presence in soil being closely associated with their host plants.

The role of mycorrhiza formation in the growth of forest trees continues to attract attention (96). The phycomycetoid type (*Rhizophagus*) occurs commonly in soil, forming associations with most plants (97). In the case of orchids, it seems to be well established that the major function of the fungi consists in providing organic substances for the growing seedling (98). In the case of other formations, the increase in root permeability is said to be of importance (99). In the case of *Rhododendron*, the endophyte was considered as a feeble parasite and of no importance to the plant (100).

The species concept of actinomycetes continues to be one of the most important problems in the study of this group of organisms (101). Their relation to the bacteria has already been emphasized (83). The actinomycetes vary considerably in morphology. Under conditions of culture, these organisms produce stable races or mutants which may differ markedly from the initial culture (102). It was suggested (103) to classify the Actinomycetales into two families, the Actinomycetaceae, with four genera, and Micromonosporaceae with one genus; this system was extended to comprise four families, namely Mycobacteriaceae, with three genera, Proactinomycetaceae, with two genera, Actinomycetaceae, with two genera and five subgenera, and Micromonosporaceae, with one genus and three subgenera. The acid-fast *Mycobacteria* have been divided (104) into three groups: (a) *Mycobact. smegmatis* type, (b) *Mycobact. phlei* type, and (c) forms more heterogeneous in nature, surviving, at 60° C., for one hour.

Whereas the soil types belong largely to the genus *Actinomyces* (105), the thermophilic population of composts consists predominantly of *Micromonospora* species (6). Distinct physiological differences seem to exist between pathogenic (potato scab) and saprophytic actinomycetes; the former use sucrose and raffinose more readily, produce pigments on tyrosine and are more sensitive to alkalis (106). Certain actinomycetes forming aerial mycelium are capable of producing porphyrin from chlorophyll (107). The antagonistic relationships of actinomycetes towards other soil microorganisms have also attracted attention (108). *Actinomyces alni* was found to be the only species capable of producing nodules on various alder plants (109), although the true significance of this association may hardly be considered to have been settled.

Protozoa and other invertebrates.—Our knowledge of the protozoan population, notably that of desert soils, raw-humus forest soils, and pastures, is increasing (110). The possible relation of some of the soil amoebae to mycetozoa has already been mentioned (89). The protozoa were shown (111) to excrete the nitrogen of the ingested bacterial cell as ammonia. As to the possible role of protozoa in modifying bacterial activities in soil, it has been admitted (112) that, in spite of their ability to reduce the numbers of bacteria, the amount of chemical work done by these bacteria need not be correspondingly reduced.

Green manures and other plant materials stimulate the development of free-living nematodes (113) and of fungi (90) capable of bringing about the destruction of plant-parasitic nematodes. Forest fires exert a change upon the soil fauna (114). The abundance and distribution of insects and other small animal forms in soil is also attracting wide attention (115); the destruction of some important injurious forms like the larvae of the Japanese beetle, by soil inhabiting bacteria, fungi, and nematodes offers great economic possibilities.

Higher plants and soil microorganisms.—The soil is known to harbor an extensive population of plant parasitic fungi and bacteria; the former comprise (116) fusaria, rhizoctonia, numerous wheat-root-infecting fungi, tobacco-infecting organisms, various bacteria some of which are said to cause the little leaf or rosette of fruit trees (117), actinomycetes causing potato scab (118), viruses of tobacco (119), and bacteriophage of legume bacteria (23).

The root system of plants exerts a marked effect upon the specific soil micropopulation (4); the latter is frequently designated as the microorganisms of the rhizosphere (120). Ecologically different plant associations show differences in the composition of the soil microflora (121). In some cases, these associations were found (122) to stimulate the germination of seeds of higher plants. Some soil bacteria seem to exert a protective effect upon certain plants against parasitic fungi; the treatment of the seed with protective bacteria was designated as bacterization (123). It still remains to be determined how beneficial such treatment is.

The favorable effect of soil microorganisms upon plant growth is, aside from the role in the transformation of organic residues and in rendering the nutrient elements available for plants, at least three-fold: (a) symbiotic relationships, as in legume symbiosis and mycorrhiza formation; (b) antagonistic action of soil organisms against plant and animal pathogens, and (c) formation of growth-promoting

substances by the soil microorganisms. The formation of the latter has been ascribed either directly to bacteria (124) or to the products of decomposition of animal manures and other plant residues (125).

The specific interrelations among different groups of microorganisms in soil, especially the bearing that this may have in repressing the development of root-infecting fungi and the relation between bacteriophage and disease production, have received in recent years considerable attention (108, 126). The disappearance of resting mycelium of *Ophiobolus graminis* in soil was found to be largely a result of the activities of the normal population of the soil; the destruction of the mycelium was favored by the addition of nitrogen-poor plant materials, thus suggesting the probability that the former serve as sources of nitrogen for the soil population (127). Furthermore, the carbon dioxide produced by the roots and microorganisms about the roots was believed to be inhibitive to development of the parasitic fungus. Various soil microorganisms, notably species of *Penicillium* were found to be antagonistic to species of *Pythium* which parasitize grasses and alfalfa (128). Certain species of *Actinomyces* were shown to be inhibitive to all fungi (129). Species of bacteria, such as strains of *B. subtilis*, of *Serratia marcescens* (129), and *B. simplex* are antagonistic to fungi; the latter was found (130) to produce a diffusible, heat-stable substance inhibiting the growth of *Rhizoctonia solani*.

It has been suggested (131) that soil saprophytic microorganisms be used to combat pathogenic fungi. The inoculation of soil with *Trichoderma lignorum*, either directly or with the manure, was found to have a highly favorable effect upon crop yields and upon the physical condition of the soil (132). It has been recently concluded (133), however, that, although organic amendments produce striking changes in the micropopulation of the soil mass, comparatively stable micropopulations are associated with the crop roots. This stability makes questionable the possibility of protecting the root surfaces from parasitic invasions by inoculation with saprophytes.

Modification of soil population and soil inoculation.—Among the various procedures for modifying the soil microbiological population, the use of heat and volatile antiseptics is of primary importance; the nitrogen-fixing and nitrifying elements are most sensitive to these treatments (134). Protozoa and nematodes are readily destroyed (135). The treatments result in an increase in soil fertility, as can be demonstrated by the increase in available minerals and organic substances (136). The introduction of copper salts results in a series of

changes characteristic of partial sterilization, first an injury to the micropopulation, followed by a return to normal (137). Most of the antiseptic substances added to the soil, whether for soil sterilization or as herbicides, tend to be destroyed by the soil micropopulation (138).

Soil inoculation with specific microorganisms, either lacking in soil or present in a less effective state, is now known to be of general importance only in the case of legume bacteria and mycorrhiza fungi. Some strains of bacteria are known to be more effective with certain legume hosts than other strains (139). In the case of plants carrying bacterial strains subject to lysis by phage, efficient strains have been selected (140). A very unstable factor was found in the sap of leguminous plants which seems to influence the extent of oxidation of the carbonaceous compounds; it also inhibits the growth of all except the homologous strains of *Rhizobia*; this factor may offer a possible explanation for the existence of cross-inoculation groups (141). The ability of a given bacterial-plant symbiosis to fix nitrogen was found to depend not only upon the strain of bacteria used, but also upon the species or even the species variety of host plant involved (139).

The ability of *Azotobacter* to become established in the soil as a result of inoculation is still open to question; it has been claimed that when accompanied by fungi, during the decomposition of straw, the introduction of *Azotobacter* into the soil will result in nitrogen fixation (142).

Determination of plant nutrients in soil.—The use of microorganisms, especially *Azotobacter*, *Aspergillus niger*, and *Cunninghamella*, for determining the plant nutrients in soil continues to arouse interest (143). However, the available elements in the soil are not constant magnitudes, but depend upon seasonal changes, as controlled by biological agents (144). The *Asp. niger* method has been utilized for measuring the amount of available copper in soil by reason of the specific requirement of the fungus for copper (145). The crop producing capacity of the soil has also been measured by the production of carbon dioxide from a mannitol-treated soil and by other procedures (146). By the use of proper soil amendments, such as magnesium ammonium phosphate, microscopic colonies were found to be capable of developing from the flora naturally present in the soil (147).

Microorganisms and soil conservation.—The losses of soil fertility as a result of cultivation and frequently of the soil itself due to water and wind erosion have recently attracted considerable attention. The microorganisms exert a threefold effect in conserving the soil: (a) by

the mechanical binding of the soil through the mycelial bodies of the fungi and actinomycetes; (b) the physical or chemical cementing of the soil particles through the mucins and other slimy substances produced by bacteria; (c) the binding effect of various decomposition products of plant and animal residues in soil. The formation of gelatinous masses and mucus materials by soil microbes has been demonstrated (148). These gelatinous materials, as well as the extensive mycelium of soil fungi and actinomycetes, exert a decided binding effect upon the soil particles, preventing their rapid removal by flowing water (132, 149).

Microorganisms and soil organic matter.—An extensive literature continues to accumulate on the decomposition of plant and animal residues by microorganisms and on the origin and nature of soil humus to which these organisms contribute through their activities and their cell substance (150). These investigations embrace a variety of problems on the decomposition of plant materials as a whole, especially green manures, composts, stable manures, etc.; the decomposition of specific plant constituents, such as cellulose and hemicelluloses, proteins and lignins; the liberation of essential elements, notably the carbon, nitrogen and phosphorus in available forms; the influence of environmental conditions, such as moisture supply, aeration and reaction, upon the decomposition processes. In these and in many other processes soil microorganisms play highly significant roles.

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ORGANIC ACIDS OF PLANTS¹

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Although malic, citric, and oxalic acids have been known since the time of Scheele, and tartaric acid, at least in the form of its salts, probably much longer, the exact position of these widely distributed substances in the general scheme of metabolism in plants is still a matter of speculation and debate. One or more of these acids is probably present in all plant tissues in appreciable and often in very considerable quantities, but in addition there are a great many other acids, which usually occur in only moderate amounts or even in traces, the relationships of which are only gradually becoming understood.

In a previous review of this field prepared in 1936 (1), Bennet-Clark pointed out the unsatisfactory nature of many of the hypotheses of organic acid metabolism that have been advanced. It was also made clear that many of the experimental methods that have been employed for the determination of these substances are open to serious criticism. Accordingly little need be said here of these matters save to emphasize further the necessity for the use of specific methods of analysis and of caution in the interpretation of the results.

INDIVIDUAL ORGANIC ACIDS

Oxalic acid.—Allsopp's hypothesis (4) that oxalic acid arises from glucose, during fermentation by *Aspergillus niger*, by the splitting off of the last two carbon atoms of fructuronic acid, leaving a four carbon atom residue for further oxidation, was mentioned by Bennet-Clark (1) in advance of publication. Allsopp found that if any of a number of organic acids, e.g., malic, succinic, pyruvic, etc., which may be regarded as possible intermediates in glucose oxidation is fed to the organism at acid reaction, no oxalic acid is produced; on the other hand, if gluconic acid or various sugars are fed at acid reaction, oxalic acid is produced. Bernhauer & Slanina (5) had observed that oxalic acid is produced from sodium formate, although not from the free acid, and Jacquot (6) now points out that oxalic acid formation is

¹ The present review includes mention of papers published previous to October, 1939, and is supplementary to the survey of the field in Volume VI (1). Attention is also directed to earlier comprehensive reviews of Bennet-Clark (2, 3).

prevented at pH 2, but is favored at pH 5 on ordinary media. A similar observation is recorded by Kirsanova (7).

The wide distribution of oxalic acid in green plants is attested by observations on its occurrence in the coffee fruit capsule (8) and in the leaves of barley, maize, oats, rye (9), spinach, broccoli, and lettuce (10).

Malic acid.—Harris & Poland (11) have studied the increase and subsequent decrease of the concentration of *l*-malic acid in the banana fruit during ripening and maturation, and Gènevois (12) has investigated the behavior in the grape. He regards malic acid formation as the result of an aerobic process dependent on respiration (see below). Donen (13) has studied the distribution of organic acid, calculated as malic acid, in the fruit of the Kelsey plum. Pucher, Clark & Vickery (14) have demonstrated that the malic acid found in all parts of the rhubarb plant is exclusively the levo-isomer; this is not in accord with the statements of Ruhland & Wetzel (15) who maintained that *dl*-malic acid is also present under certain conditions.

Citric acid.—A review of the citric acid industry has been published by Wells & Herrick (16). The production of citric acid by microorganisms, especially *Aspergillus niger*, has been extensively studied. Chrzaszcz & Zakomorny (17) have investigated the so-called physiological degeneration and regeneration of molds in culture under certain conditions as they affect the citric acid produced. Johnson, Knight & Walker (18) have discussed the mechanism of formation of citric acid from sugar and have pointed out, in view of the failure of iodoacetic acid to suppress citric acid formation, that the classical alcohol fermentation reactions do not precede citric acid synthesis, and in this they are supported by Barinova & Butkevich (19). Various mechanisms to account for citric acid synthesis in molds have been put forward by several investigators (6, 20, 21, 22), and an interesting and authoritative review of this topic is given by Wells, Moyer & May (23).

Citric acid synthesis by green plants has attracted much attention. The possibility of its commercial production from, especially, *Nicotiana rustica* has been considered in Russia, and several papers on citric acid in this species have appeared (24, 25, 26, 27). *N. glauca* is also rich in citric acid² and may become of commercial importance

² It is often difficult to tell whether the statements regarding citric acid in these Russian papers refer to the fresh leaves or to cured or even fermented leaves. The high proportion of citric acid mentioned suggests, however, that cured leaves are frequently meant.

not only on this account (28), but also because it furnishes a source of the alkaloid anabesine which may be used as an insecticide as a substitute for nicotine. [See also (29, 30) for a discussion of hybrids in this connection.]

Citric acid has been identified in the pineapple fruit (31), in the fruit of the cannonball tree, *Couroupita guianensis* Aubl. (32), and in the potato tuber (33).

Probably the most significant work on the metabolism of citric acid is that of Martius (34). Although this work deals chiefly with the metabolism of citric acid in animals—a theme reviewed elsewhere in this volume (pp. 18, 269)—it applies directly to plants as well, and is more fully discussed in another connection below.

Isocitric acid.—Known previously only as a product of synthesis (36), this optically active isomer of citric acid was first reported in nature in 1925 by Nelson (35) who found that it is the chief organic acid of the blackberry fruit. It has recently been obtained by Nelson & Wheeler from cannonball tree fruit (32), it has been studied by Bruce (37) in some detail, a new synthesis has been given by Greenstein (38), and it occupies an important position in the scheme of citric acid metabolism suggested by Martius & Knoop (39) and by Martius (34).

Aconitic acid.—Citric and isocitric acid are closely related to aconitic acid, into which they can be converted merely by the loss of the elements of water with the formation of a double bond. Aconitic acid has long been known in nature (see 40). A convenient method for its preparation has been given by Bruce (41), and a method for its determination by Johnson (42). The presence of an enzyme in cucumber seed (42) and in pigeon muscle (42, 43) specific for the reversible hydration of *cis*-aconitic acid suggests the significance of this substance in metabolism, and a hypothetical interpretation of its position is given in the papers of Martius [(34) see below].

Other acids.—Itaconic acid, which can be regarded from the point of view of structure as a product of decarboxylation of aconitic acid, has been found by Calam, Oxford & Raistrick (44) in the culture solution in which a special strain of *Aspergillus terreus* had grown. The yield from the glucose metabolized was of the order of six per cent. This is only the second record of the occurrence of this substance in nature.

The fruit and leaves of *Bauhinia reticulata* D.C. have been found by Rabaté & Gourévitch (45) to contain five per cent or more of

l-tartaric acid. Górski (46) has studied the utilization of the optical isomers of tartaric acid by *Aspergillus fumigatus* Fres. Unlike *A. niger*, this organism utilizes the dextro-isomer completely when grown on a culture solution that contains racemic acid; the utilization of the levo-isomer was small. Variation in temperature or concentration, or the addition of salts of iron, manganese, calcium, strontium, or uranium, had no effect. The rate was depressed by addition of boric or molybdic acid and was stimulated by addition of copper salts. The rate was also accelerated materially if *l*-malic acid was added. If the organism was grown on *dl*-malic acid, both isomers were used, although the *d*-isomer disappeared somewhat more rapidly than the *l*-isomer.

An enzyme which oxidizes dioxymaleic acid specifically has been noted in horse-radish root by Banga & Szent-Györgyi (47), and also in paprika fruit, onion roots and leaves, asparagus shoots, and other plant tissues (48, 49). The substance is a hypothetical intermediate in the oxidation of succinic acid. The literature of fumaric acid in mold metabolism has been discussed by Foster & Waksman (50, see also 51, 52, 53, 54). The occurrence of lactic acid in higher plants has been studied by Schneider (55).

Virtanen & Laine (56) claim to have demonstrated the presence of oxalacetic acid in the pea plant and advance the view that the nodule bacteria receive this substance by way of the roots and employ it in the synthesis of aspartic acid which can then enter into the nitrogen metabolism of the host. In a later paper, Virtanen, Laine & Roine (57) describe a new method to detect oxalacetic acid in plant tissue and confirm their earlier observation (see also 58). Nevertheless attempts by Wyss, Burris & Wilson (59) to verify these findings were not successful.

Quinic acid has assumed some significance in speculations on the conversion of sugar to citric acid (22) and the closely related substance shikimic acid is held by Fischer & Dangschat (60) to be related in an analogous manner to aconitic acid and thus to tricarballic acid. The synthesis of this last substance has been studied by Greenstein (38).

α -Ketoglutaric acid has been demonstrated in pea seedlings by Damodaran & Nair (61) by isolation as the dinitrophenylhydrazine derivative. This substance is the oxidation product of glutamic acid formed by a dehydrogenase present both in plants and animals (62). Together with oxalacetic acid, it is of great importance in connec-

tion with recent views of amide metabolism in plants (63, 64). A convenient method of synthesis is given by Weil-Malherbe (65).

ANALYTICAL METHODS FOR ORGANIC ACIDS

The study of the metabolism of organic acids in plants has now reached a phase in which further progress can be anticipated only if accurate and specific analytical methods for the various acids are commonly employed. The day is now past when the results of a simple titration may be calculated in terms of malic or citric acid and interpreted in any meaningful way. It is impossible in the space available to deal adequately with recently published analytical methods of direct concern to plant biochemistry, but the following references may serve as a key. Several important methods described prior to 1937 have been mentioned by Bennet-Clark (1) and the present list is merely supplementary to his discussion: oxalic acid (66, 67); citric acid (68, 69); malic acid (70, 71); aconitic acid (42, 72); lactic acid (73, 74); succinic acid (53, 75); tartaric acid (76); oxalacetic acid (52, 57, 59, 77, 78); α -ketoglutaric acid (79, 80); pyruvic acid (81); gluconic acid (82); aspartic acid (method of importance in connection with the determination of oxalacetic acid) (83); volatile fatty acids (84, 85).

The general method of distillation of the esters of organic acids, employed in the classical work of Franzen [see bibliography of (1)], is still of fundamental importance for qualitative investigation, and it has been found in the laboratory of the writers that this procedure can be so conducted as to yield surprisingly accurate quantitative results (86). Accordingly, the method described by Micovic (87) of esterification in the presence of toluene, in which water is removed by distillation of the azeotropic mixture, is of interest. The use of dihydroxyfluoboric acid as a catalyst (88) has also been described. It will perhaps not be out of place to call attention in this connection to the volatility of oxalic acid from hot solutions. Some extraordinary observations have been described by Howard (89). An almost complete loss of oxalic acid, during esterification of the mixture of acids present in tobacco leaves, was experienced by Vickery & Pucher (86). The material they employed was rich in this substance, but only traces of the ester were found after esterification by the usual technique.

Mention may also be made of a study by Archibald (90) of the distribution of organic acids between water and a number of immiscible solvents, and of a convenient type of extraction apparatus for use with plant organic acids (91).

ACID-BASE RELATIONSHIPS

One of the obvious functions of organic acids in plant tissues is to provide a buffer system for the protection of the cells against large fluctuations of hydrogen ion concentration. The concentration of, for example, malic acid in such tissues as tobacco leaf or rhubarb petiole is usually in the range 0.1 to 0.2 *M*, calculated on the assumption that all of the acid is present in solution in the entire mass of water present. This is certainly an underestimate of the true physico-chemical conditions and it is clear that, at the pH reactions of plant cell saps, the buffering capacity due to the organic acids alone, without consideration of the amino acids, proteins, and possibly the phosphates, must be considerable.

Attention has been given to the relative proportions of acidic and basic constituents in samples of fermented tobacco leaves by Pucher, Vickery & Wakeman (92) who have shown, from complete analyses of the ash for inorganic components as well as of the tissues for organic acids, that the large excess of positive ions present is closely correlated with the total organic acidity. The organic acids occupy a dominating position with respect to the balance of positive and negative ions, and it is held that these substances are closely concerned in the phenomena of inorganic nutrition.

Wadleigh & Shive (93) have determined the organic acids of the maize plant in specimens grown in culture with the continuous flow technique at a series of different reactions (pH 3.0 to 8.0), and found that the effect upon these substances of the form in which nitrogen is furnished is greater than that of the reaction of the medium. Plants supplied exclusively with nitrate contained more organic acid (as well as more inorganic base) than those supplied with both ammonium and nitrate nitrogen in approximately equal amounts. This observation confirms that of Clark (94) with the tomato plant, and experience with tobacco in the writers' laboratory (unpublished) indicates that it is probably a general phenomenon. Not only the total amount of organic acid, but the relative proportions of the different acids present, are profoundly affected by the form in which nitrogen is administered to the plant.

"CRASSULACEAN" METABOLISM

The so-called Crassulacean type of organic acid metabolism derives its name from observations made many years ago (95) with leaves of *Bryophyllum* and other succulent plants of the family *Cras-*

sulaceae. In these species there is a large diurnal variation in titratable acid content. The loss of acid during the day and the gain during the night were attributed to changes in the malic acid and were associated by Kraus (96) with fluctuations of the carbohydrate concentration in the reverse direction.

This phenomenon has received a great deal of attention (2), and modern methods of organic acid analysis are now being applied. Wolf (97) has found that diurnal variations occur not only in the malic acid, but also in the citric acid and in the group of unknown acids that make up a relatively small part of the total organic acidity. Guthrie (98) finds that the change in citric acid may account for as much as one quarter of the change in acidity, and Borgström (99) likewise emphasizes the importance of citric acid in this connection.

Pucher, Wakeman & Vickery (100) have studied the organic acid composition of the buckwheat, a nonsucculent plant which shows a *small* diurnal variation in acidity (pH) as contrasted with the large variation observed in *Bryophyllum*, *Sedum*, and *Opuntia* species. They find that the changes cannot be accounted for simply in terms of variation in the quantities of malic and other acids present since a decrease in hydrogen-ion activity may accompany an actual increase in acid. They point out that such factors as the absorption of inorganic ions by the roots, the distribution of organic and inorganic bases within the tissues, and photosynthesis may all play a part in the small changes in acidity observed in plants of this type.

Thoday & Jones (101) have studied malic acid metabolism in *Kleinia articulata*, a succulent member of the family *Compositae*. Diminution in malic acid content during starvation of the leaves in darkness was observed (see next section) as well as daily fluctuation in acid content. They were unable, however, to demonstrate a similar and parallel fluctuation in respiration rate as had been found by Bennet-Clark in *Sedum* leaves (2).

TOBACCO PLANT

Analyses of fresh tobacco leaves, cultured in water or in nutrient solutions either in continuous light or in continuous darkness, have been made in the writers' laboratory (102, 103). The data embrace determinations of the individual organic acids and of the carbohydrates and nitrogenous constituents as well. It was found that the total organic acidity changed but little, there being evidence for only a slight increase in light and a slight decrease in darkness. The oxalic acid

remained substantially unchanged in either case, but there was a wide difference in the behavior of the malic and citric acids under the two conditions. In light these two acids changed very little, but in darkness the malic acid diminished rapidly, the loss being one of the largest individual changes that was observed. At the same time the citric acid increased materially, and the changes were interpreted to signify a conversion of malic to citric acid, either direct or indirect, under these conditions of leaf culture.

A similar study of the behavior during culture of tobacco stalks denuded of leaves (104) led to somewhat different results. The total organic acidity increased slightly at the start, both in light and in darkness, and then diminished slowly. Oxalic acid did not change. Citric acid, which is present in only small amounts, increased significantly in darkness, but diminished slightly in light. Malic acid, however, which is present in considerable quantity in tobacco stalks, increased both in light and in darkness, though more slowly under the latter conditions. The definite difference between the organic acid metabolism in tobacco leaves and in tobacco stalks is doubtless an expression of the wide differences in composition. Whereas the leaves are relatively high in protein and low in carbohydrates, the reverse is the case for the stalks.

Reference to a number of Russian investigations on the organic acids of various species of tobacco has already been made. Mikhlin & Bakh (105) have recently carried out the interesting experiment of infiltrating neutral solutions of oxaloacetic, pyruvic, or malic acid into young tobacco leaves and have observed a prompt and extensive synthesis of citric acid. Other more general papers on the organic acids of varieties of tobacco plants are those of Kovalenko (26), of Sirotenko (106), and of Darkis, Dixon, Wolf & Gross (107).

RHUBARB PLANT

The rhubarb plant has long been recognized as a valuable species for the study of organic acid metabolism (see 1). Pucher, Clark & Vickery (108) have examined the organic acid composition at successive stages of development and find wide differences in composition in leaves of different physiological age. The group of unknown acids, i.e., total organic acids minus the sum of the oxalic, malic, and citric acids, predominates in the blades of young leaves, oxalic acid being present in only slightly smaller amount. In blades of leaves of later and fuller development, oxalic acid predominates, *l*-malic and citric

acids being present in relatively small amounts. In the petioles, however, *l*-malic acid is the predominating acid, oxalic acid occurs in lesser amounts, and citric acid and the unknown acids in only small quantities. There is evidence for a gradient in concentration of acids from leaf blade to veins to petiole; the concentration of oxalic, citric, and unknown acids decreases in this direction, while that of malic acid increases with the result that the concentration of total organic acids is not far from constant in all parts of the leaf. No correlation between any of the organic acids and the ammonia content was found.

In a later publication, Pucher, Wakeman & Vickery (109) record the behavior of the organic acids of rhubarb leaves during culture in water or in glucose solution, both in light and in darkness. The full data of this investigation, including the carbohydrates and nitrogenous components, are given in a more comprehensive publication (63) in which theoretical interpretations of organic acid metabolism are also discussed. It was found that the behavior of the acids of rhubarb leaves differs in many respects from that of the acids of tobacco leaves. There is a diminution in total organic acids during culture in darkness, probably due to utilization of part of the acids in respiration. In light there is a temporary increase due almost entirely to an increase in malic acid. Culture in glucose solution brought about similar but more prolonged and extensive changes, and the evidence strongly suggests that a transformation of some of the imbibed carbohydrate into malic acid occurred. Neither citric nor oxalic acid underwent extensive change. There was no close correlation between the change in the hydrogen-ion activity of the leaves during culture and the changes in organic acids; furthermore the marked increase in ammonia was quite insufficient to account for the decrease in acidity. A far-reaching reorientation of acidic and basic components must therefore have taken place.

Allsopp (110) has considered the organic acid metabolism in the rhubarb plant from the standpoint of seasonal change. Both roots and tops of the plants were studied throughout a growing season, and the fluctuations in concentration and in the actual amounts of the several acids per plant were recorded. Transport of acids from the rhizomes to the leaves occurred during the early growth period, so that ultimately the concentration in the young leaf exceeded that in the rhizome. Later all acids increased as a result of metabolism of the products of photosynthesis, and transport of acids back to the roots for storage became increasingly important as the season pro-

gressed. The acids are clearly demonstrated to play an active role in the metabolism of this plant, although their relationship to the other main components of the tissue still escapes precise definition.

HYPOTHESES OF THE METABOLISM OF ORGANIC ACIDS

The close relationship between the organic acids of plants and the amides, asparagine and glutamine, has been emphasized by various workers for nearly a century; (for literature see 64). The synthesis of amides in plants has been held by Prianischnikow (111) to represent a more or less obligatory mechanism whereby ammonia is converted into a neutral nontoxic substance by combination with some suitable organic acid. The probability that the immediate precursors of the two common amides, asparagine and glutamine, are respectively oxaloacetic acid and α -ketoglutaric acid, has been pointed out by the writers and their associates (102), and a hypothetical mechanism whereby these substances may arise in plant tissues has been suggested by Chibnall (112). According to this view, the reactions involved in the citric acid cycle proposed by Krebs & Johnson (113) to account for the respiration of carbohydrate in muscle tissue, may be applied to the respiration of plant tissues. Martius (34) and Martius & Knoop (39) have shown that certain of the reactions in this sequence are catalyzed by enzymes present in liver. These reactions are the condensation of pyruvic acid, derived from the carbohydrate, with oxaloacetic acid to form a product which, on being oxidized and decarboxylated, yields citric acid, and the successive transformation of this to aconitic acid, isocitric acid, oxalosuccinic acid, and finally to α -ketoglutaric acid. α -Ketoglutaric acid may then be oxidized to succinic acid by the Warburg-Keilin system, and this in turn transformed through fumaric and malic acid back to oxalacetic acid, by a series of reversible enzymatic reactions established by Szent-Györgyi and his co-workers (52). The combination of these sequences of reactions into a cycle provides a metabolic scheme whereby carbohydrate enters by way of pyruvic acid, oxygen enters at the transformation of α -ketoglutaric acid to succinic acid, and carbon dioxide is eliminated by decarboxylation reactions at several points. Each of the components of the system has been shown to stimulate the respiration of various animal tissues, and many of the transformations have been demonstrated to be brought about by enzyme systems present in both plants and animals. Furthermore, with one exception (oxalosuccinic acid), every intermediate considered is more or less well known as a component of plant tissues, particularly of leaves.

Chibnall has added to this scheme formulations to illustrate the possible combination of the α -keto acids with ammonia to produce α -amino acids (see 114), and the subsequent conversion of aspartic and glutamic acid respectively to asparagine and glutamine by well known enzymatic reactions. Steps are also indicated whereby fats and amino acid residues may enter through succinic acid, and proteins by way of α -keto acids. The entire hypothetical system furnishes a basis for the discussion of respiration as well as of different phases of protein metabolism in terms of the organic acids commonly found in plants. The organic acids are thus placed in a rather clearly defined relationship to the three main groups of plant constituents, the proteins, the carbohydrates, and the fats. Whether or not the actual relationships are in detail even as simple as this admittedly complex mechanism would indicate is immaterial at the moment. The point is that the metabolism of the organic acids is definitely linked with the respiration of the tissues, and it remains for future investigation to clear up the subsidiary points presented by the individual transformations.

One step in this direction has been attempted by the writers (63) in connection with their studies of rhubarb leaves. A careful consideration of the composition of the tissues at different stages of culture indicated that no observation in conflict with Chibnall's view had come to light. This is perhaps not surprising since the very flexibility of such elaborate schemes renders proof one way or the other exceptionally difficult. However, a reasonably satisfactory general account of amide metabolism in terms of respiration of the tissues can now be given (64), and the overemphasis on the function of ammonia in amide synthesis, implied in the "detoxication" view of Prianischnikow, is removed. The interesting observations of Mikhlin & Bakh (105) already mentioned may also be cited as evidence for the possibility of organic acid interconversions in plant tissues in line with this metabolic scheme.

One other attempt to account for the function of the organic acid in plants, again in connection with respiration, should be mentioned. Gregory & Sen (115), as the result of careful statistical analysis of their comprehensive data on the respiration and composition of the leaves of barley, have reached the conclusion that the leaf protein is in some way involved in respiration. A highly significant positive correlation between the respiration rate and the protein content was found, notwithstanding the wide variations in composition brought

about by culture of the plants under different degrees of deficiency of potassium, nitrogen, or phosphorus (116). In order to explain this relationship, Gregory & Sen suggest the possibility that the protein of the cells is related to its products of digestion by a series of highly generalized reactions arranged in a cycle, according to which protein digestion and protein synthesis follow different paths. The products of glycolysis are drawn into this cycle directly, or through intermediary organic acids, and these can combine with ammonia, produced by oxidation of amino acids or derived from the soil, to form amino acids and amides. In addition, the residues of oxidative deamination of amino acids may be further oxidized to provide energy. The scheme thus provides for a supply of organic acids from carbohydrate oxidation, these in turn furnishing material for protein synthesis, while protein degradation products also contribute to the organic acids. The cycle of protein synthesis and decomposition is held to be rapidly traversed, and its demands upon the organic acids, and, in turn, upon the carbohydrates regulate the rate of glycolysis. The output of carbon dioxide is thus related to the rate at which the protein is decomposed and regenerated, but the carbon dioxide evolved may or may not originate chiefly from the oxidation of the amino acids. In general it would seem likely that quotas may be provided from several sources.

The essential feature of the system is that these several processes are interrelated and connected by some general regulatory mechanism, and thus this view shares, with the more specific mechanisms envisaged by Chibnall, the notion that the organic acids occupy a definitely intermediary relationship between the carbohydrates and the proteins. They are doubtless also connected in some similar fashion with products of fat oxidation, which must occupy a dominating position in such cases as the metabolism of many species of seedlings.

An examination of data obtained in the course of their study of rhubarb leaf metabolism enabled the writers to test one aspect of these views of Gregory & Sen. It was found (117) that respiration under certain conditions could be demonstrated to draw upon the protein of the leaf blade as a source of some of the carbon lost from the tissue and, even when glucose was supplied in the culture solution or when the leaves were exposed to light, there was evidence that some of the protein decomposition products were still involved. Accordingly these views, even though expressed only in a highly generalized form without specification as to the exact nature of the

chemical reactions, hold forth hope that some progress has been made in the solution of the problem of respiration. It is highly significant that the plant organic acids should occupy the central position in all attempted theoretical treatments of the subject.

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THE BIOCHEMISTRY OF VIRUSES

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Viruses were discovered in 1892 but their biochemistry is a relatively new subject. It was not until 1935 that a tangible, characteristic material carrying virus activity became available for biochemical study and the nature of viruses was indicated fairly clearly. Previously, viruses had been recognized only by means of their biological activity and although there had been much work on the effect of different treatments on viruses, opinions concerning their nature were extremely varied. It should be mentioned that vaccine virus was purified and isolated as early as 1922, but since the elementary bodies of viruses of this type are similar in appearance and size to accepted organisms, their purification had but little influence on the trend of biochemical investigations of viruses in general. A stimulus was provided by the chemical isolation of a high molecular weight crystalline protein material possessing the properties of tobacco mosaic virus (154). A widespread search for similar materials in both animal and plant tissues diseased with different viruses followed and has resulted in the discovery during the past four years of over twenty different materials of high molecular weight possessing some of the properties of the respective viruses or virus strains. The volume of this work is increasing at a very rapid rate, yet to date the investigations on most of these materials have been of a somewhat fragmentary nature. However, tobacco mosaic virus has been the subject of an extremely varied and most extensive series of researches. In addition, the purified preparations of certain strains of tobacco mosaic and the related cucumber mosaic -3 and -4 viruses, of latent mosaic of potato, Shope rabbit papilloma, bushy stunt and tobacco ring spot viruses, and of a staphylococcus bacteriophage have been subjected to considerable investigation. Some of this work has been described in previous volumes of this *Review*. The isolation of tobacco mosaic virus was mentioned in the *Review* for 1935 (124) and the immunological results obtained with purified active and inactivated virus and the first sedimentation analyses were described in the *Review* for 1936 (1, 72). The following year, Bergmann & Niemann (15) reviewed three important advances, the isolation of the same characteristic protein of

high molecular weight from various, in some instances unrelated, species of mosaic-diseased plants, the isolation of different strains of tobacco mosaic virus in the form of slightly different, although closely related proteins, and the isolation of several other viruses in the form of protein materials possessing quite different and characteristic properties. In last year's *Review*, Astbury (2) gave a stimulating presentation of the x-ray work on the viruses and Tiselius (171) mentioned the isolation of bushy stunt and the cucumber mosaic viruses and described some of the more important work on the pH stability range, aggregation, inactivation, reactivation, viscosity, and diffusion of tobacco mosaic virus. Since the virus researches described in the previous volumes of this *Review* have been adequately covered, an attempt will be made in the present *Review* to present work published during 1939 together with a few papers which appeared earlier but which did not fall within the province of the earlier discussions. Publications pertaining to purely pathological or physiological aspects are not included. The appearance of several general reviews (4, 19, 21, 26a, 52, 52a, 71, 75, 81, 84, 85, 94, 95, 97, 102, 123, 125, 156, 158, 161, 162, 164, 165, 178), of 5 books (5, 28, 86, 129, 132) and of a new journal (27) devoted to viruses should be noted. The reviews by Blunck (21), a botanist; Levaditi (84), a pathologist; Lynen (94), a chemist; McFarlane (97) and Wyckoff (178), biophysicists, are especially recommended. Accounts of progress in various phases of virus research are also to be found in the *Proceedings of the Third International Congress for Microbiology*, New York, and the *Proceedings of the Fourth International Congress for Comparative Pathology*, Rome.

Isolation methods.—Tobacco mosaic virus was first isolated by a procedure involving the salting out methods used so successfully by Northrop and co-workers for enzymes (108). Advantage was also taken of the fact that the virus could be adsorbed on a material such as celite at acid reactions and eluted at alkaline reactions. These same general methods (28, p. 477) together with the occasional use of acetone precipitation, trypsin treatment, or the judicious use of heat have been utilized in the purification of four different strains of tobacco mosaic virus (5, 112, 114, 126, 155, 170), two related viruses (5), bushy stunt of tomato (7), latent mosaic of potato (6, 90), tobacco necrosis (116), and potato "Y" (9) viruses and a staphylococcus bacteriophage (107). The efficacy of these strictly chemical methods became questionable when it was found that unless the isolations were

carried out rapidly, carefully, and in the cold, the purified preparations of tobacco mosaic virus consisted of aggregated, partially inactivated virus which had lost one of the primary characteristics of a virus, namely filtrability through membranes of small pore size (5, 92, 148, 158). Loring (90) found that five successive precipitations of latent mosaic virus with ammonium sulfate in the cold caused a 50 per cent reduction in activity and that even one precipitation with ammonium sulfate at room temperature resulted in a demonstrable loss of activity. Similarly, one precipitation of tobacco ring spot virus with ammonium sulfate at 4° C. was found to cause a 50 per cent reduction in activity and contact with 30 per cent ammonium sulfate for three days at 4° C. caused almost complete inactivation (159). Bawden & Pirie (9) noted that the chemical methods which they used for the purification of potato "Y" virus caused a large amount of inactivation. It became obvious that, as was emphasized by Tiselius (171) in last year's review on proteins, less drastic methods would have to be used in order to secure unaltered and fully active preparations of some of the less stable viruses.

Fortunately, during recent years apparatus was developed, chiefly through the efforts of Svedberg, Tiselius, Henriot, Huguenard, Beams, Pickels and Wyckoff, which permitted rather large quantities of material to be subjected to intense electrical and centrifugal fields and the movement of the major components to be followed by optical means. Differential centrifugation found immediate favor as a preparative method for viruses because of their unusually high molecular weight and has been used in several laboratories (43). By means of this mild physical method purified preparations of tobacco mosaic (179) and its strains and related viruses (121, 157, 177), and of latent mosaic of potato (90), tobacco ring spot (159), tobacco necrosis (122), alfalfa mosaic (135), Shope rabbit papilloma (12), chicken tumor I (24, 25, 117, 166), equine encephalomyelitis (142, 169, 176), yellow fever (51), jaundice of silkworms (45), and fowl leucosis (54, 167) viruses have been obtained. The homogeneity of some of these preparations in the Tiselius electrophoresis apparatus has been determined (30, 98) but as yet electrophoresis has not been used as a preparative method for viruses, although with time it will undoubtedly come into use for this purpose. It should be emphasized that purified virus preparations obtained by differential centrifugation alone or in conjunction with electrophoresis should not be regarded as molecularly homogeneous for it is quite possible that a virus and an associated

impurity might move at the same rate in centrifugal and electrical fields. Such preparations should be subjected to other tests before a decision regarding homogeneity is reached. However, in the case of some of the plant viruses, particularly tobacco mosaic virus, numerous additional tests have failed to reveal the presence of an impurity in the samples prepared by differential centrifugation. The efficacy of the method in the case of plant viruses appears to be due to the fact that the viruses represent the only high molecular weight components present in the diseased plants which will withstand freezing, treatment with salt, and repeated ultracentrifugation. The proteins of high molecular weight in normal plants appear to be quite different from the virus proteins and are denatured by such treatment, especially by the freezing process (8, 93, 122). The purification procedure involving differential centrifugation is, therefore, especially well suited for the plant viruses.

Unfortunately, the situation with respect to most of the viruses affecting animals is not so happy, for, although such viruses are readily sedimented by ultracentrifugation, it has been found that similar extracts of normal animal tissues also contain material that is sedimented in the ultracentrifuge. In the cases of the chicken tumor I and equine encephalomyelitis viruses highly active preparations reasonably homogeneous with respect to rate of sedimentation have been obtained which, as yet, are otherwise indistinguishable from inactive preparations obtained in a similar manner from normal tissues (25, 141). Pollard (117) also found preparations obtained from nonfiltrable tumors to have properties very similar to those of purified chicken tumor I preparations. Additional tests are necessary in order to determine whether the active differs from the inactive preparation in certain chemical properties or the active preparation consists of a small percentage of virus plus a large percentage of the same material found in the inactive preparations. It should be mentioned that Beard, Bryan & Wyckoff (12), using methods similar to those used for the isolation of the purified rabbit papilloma virus, found no evidence for the existence of a similar material in normal tissues or in the virus-induced noninfectious papillomas of domestic rabbits. However, indirect evidence for the existence in the latter material of virus, probably in a masked form, has been obtained by Shope (144) and by Kidd (68).

Extensive work with plant viruses was delayed until accurate methods for measuring virus activity became available. It is encourag-

ing, therefore, to note that attention is being directed to this problem in connection with viruses affecting animals. Bryan & Beard (23) have described a method for the estimation of the rabbit papilloma virus by infectivity measurements. In a continuation of the work in Rivers' laboratory on measurements of the activity of vaccine virus preparations, Smadel, Rivers & Pickels (147) have found that the ratio of infective units of vaccine virus to elementary bodies is about one to four. This is the lowest ratio of biological to physical unit that has been achieved with any virus and is an indication that the present preparations are essentially pure and that one elementary body may cause infection.

Composition.—Several of the purified virus preparations have been subjected to chemical analysis and have been found to contain about 50 per cent carbon, about 7.5 per cent hydrogen, about 16 per cent nitrogen, from 0.2 to 2.2 per cent sulfur, from 0.5 to 4.1 per cent phosphorus and in the case of some of the viruses affecting animals, varying amounts of lipid. The chemical composition of the molluscum contagiosum inclusion body has also been studied (174). Since preparations of different viruses contain approximately the same amounts of carbon, hydrogen, and nitrogen, the sulfur and phosphorus contents are much more useful in the characterization of a given virus. The sulfur content of tobacco mosaic virus is usually 0.24 per cent and of this about 0.18 per cent occurs as cysteine sulfur and 0.04 per cent or less as sulfate sulfur (134). It should be recognized, however, that elementary chemical analyses have little significance unless it can be shown by other means that the preparations are essentially pure. As a matter of fact, special properties, such as coagulability and specific color tests, are much more significant and useful in the characterization of a protein than are elementary chemical analyses. Attention has been given to the lipid of the purified preparations of vaccine and chicken tumor I viruses. Attempts to remove the final 9 per cent of lipid from vaccine virus have resulted in inactivation (99). Pollard (117) obtained active purified preparations of the chicken tumor I virus containing only 5 per cent lipid by extracting the crude starting material with lipid solvents. However, trypsin treatment of these removed protein and yielded final preparations containing about 40 per cent lipid, or an amount about the same as that reported by Claude (25). Since these purified preparations were isolated from tissues containing much lipid it is possible that the lipid may represent a contaminant, yet until it can be removed

without loss of activity, lipid must be regarded as an integral component of these viruses. It does not necessarily follow that viruses affecting animals and viruses affecting plants differ with respect to their lipid content, for the small amount of lipid found in purified preparations of the rabbit papilloma virus is regarded as an impurity and not an integral part of the virus (12). A lipid contaminant has been found to be associated with one plant virus and although not separable by salting out methods it was readily separated by differential centrifugation (9). McFarlane & Salaman (100) have found that purified vaccine virus shows phosphatase and catalase activities and consider these to be specific properties of the virus.

The most outstanding and perhaps significant finding resulting from chemical analysis of the purified virus preparations is that, so far as examined, they are composed either entirely or almost entirely of nucleoprotein. In the case of tobacco mosaic virus, the material first isolated was considered to be a globulin because of its solubility characteristics and because no phosphorus was found. However, later preparations obtained by less drastic treatment were found to contain 0.5 to 0.6 per cent phosphorus. Following the isolation of crude preparations having some of the properties of nucleic acid from purified tobacco mosaic virus almost simultaneously by Bawden and associates (10) and by Stanley (155), the view was advanced by the former workers that the preparation consisted of nucleoprotein. This view has been substantiated by work from both laboratories, especially by that of Loring (91) who first isolated protein-free virus nucleic acid and established its structure by the isolation and identification of the hydrolytic products. The preparations of nucleic acid from plant viruses described by Bawden & Pirie apparently contained from about 20 to 60 per cent of protein as an impurity for phosphorus contents of only 4 to 7 per cent were reported (5), whereas nucleic acids are known to contain about 9 per cent of phosphorus. Loring (91) found 8.8 to 9.5 per cent of phosphorus in different preparations of tobacco mosaic virus nucleic acid and Stanley (159) found 9.3 per cent of phosphorus in the nucleic acid from tobacco ring spot virus. The nucleic acid components of all of the plant viruses that have been examined and of the chicken tumor I virus (25) have not given a test for desoxy sugar but have given tests for a pentose, hence they are probably of the yeast nucleic acid type. On the other hand, the elementary bodies of vaccinia and of psittacosis have been found to give a test characteristic of thymus nucleic acid (99,

130). Although this may prove of significance from the standpoint of the evolution of viruses, there appears to be no need to attach undue importance to the fact since cells are known to contain both types of nucleic acid and since, on the basis of chemical structure, thymus nucleic acid differs from yeast nucleic acid only in that in the former the pyrimidine component is methylated and the sugar reduced. Furthermore, Loring (91) has obtained evidence that the uridylic acid isolated from tobacco mosaic virus nucleic acid may be isomeric with that from yeast nucleic acid; hence, with time, nucleic acids differing slightly in structure may be found in the viruses.

Kalmanson & Bronfenbrenner (55) found a purified *B. coli* bacteriophage preparation to give the usual protein color tests and to contain 49.3 per cent carbon, 7.9 per cent hydrogen, 14.0 per cent nitrogen, 0.07 per cent phosphorus and 1.0 per cent ash and concluded that the material was a simple protein. On the other hand, Northrop (107) and Schlesinger (138) considered that their phage preparations consisted essentially of nucleoprotein. It is not known whether the difference in the results is due to differences in the degree of purity of the preparations or to the fact that different bacteriophages were used. Much of the phosphorus in Northrop's phage preparation appears to be in a form other than in nucleic acid for if all of the carbohydrate which was found occurs in the form of nucleic acid it would account for only about one-tenth of the phosphorus.

The protein components of the purified virus preparations have received but little attention, except in the case of tobacco mosaic virus. The amino acids which have been found by Ross (133, 161) in the protein component of this virus include arginine, aspartic acid, cysteine, naturally occurring *l*(+)-glutamic acid, leucine, lysine, phenylalanine, proline, serine, tyrosine, and tryptophane. Histidine, alanine, and glycine appear to be absent. Since the arginine content is only about 8.8 per cent and there is not a preponderance of other basic amino acids, the tobacco mosaic virus is hardly comparable to sperm nucleoproteins which are composed of a histone or a protamine plus nucleic acid and which have, therefore, a high basic amino acid content. Furthermore, no evidence has been obtained that the protein and nucleic acid components of the virus can be dissociated by salt solutions which are known to cause the dissociation of sperm nucleoproteins. This indicates that the linkage in this virus may be different and probably somewhat stronger than the usual salt bonds. However, the situation appears to vary from virus to virus for Loring (90)

found that the nucleic acid is bound far more strongly in latent mosaic virus than in tobacco mosaic virus, Bawden & Pirie (7) demonstrated that the linkage is even stronger in bushy stunt virus, and Stanley (159) found that a partial dissociation of the nucleic acid component of tobacco ring spot virus occurs in a 30 per cent ammonium sulfate solution. The latter virus differs from other viruses in that it has an unusually high nucleic acid content of about 40 per cent and is, in this respect, somewhat more comparable to sperm nucleoproteins.

Absorption spectra.—Ultraviolet light absorption spectrum measurements have been made on purified preparations of several viruses and in general there is an absorption maximum at about 2600 Å, a minimum around 2500 Å, and general absorption at shorter wave lengths. The results are in accord with what might be expected of materials containing nucleic acid which is known to absorb strongly at 2600 Å. The amount of the absorption appears to be a measure of the nucleic acid content, for tobacco ring spot virus with a nucleic acid content of about 40 per cent absorbs most strongly, and bushy stunt, tobacco necrosis, chicken tumor I, latent mosaic, and tobacco mosaic virus preparations with nucleic acid contents of from about 20 to about 5 per cent absorb proportionally less (5, 25, 82, 116). It may be noted that wave lengths near 2250 Å where protein absorbs strongly have been found to be more effective for inactivating tobacco mosaic virus than wave lengths near 2600 Å (49). In a study of the absorption spectra of the components of tobacco mosaic, latent mosaic, and tobacco ring spot viruses, it was found that, although the spectra of the nucleic acids were about the same, the spectra of the protein components were different and characteristic for each virus (82). It appears likely that additional work on the protein and nucleic acid components of different viruses will reveal further differences.

X-ray studies.—The x-ray studies on several viruses by Wyckoff & Corey and by Bernal and associates which were reported in last year's *Review* (2) have been extended by the latter workers (17) to include bushy stunt virus which crystallizes in the form of isotropic rhombic dodecahedra. Powder photographs of a suspension of these crystals were taken and spacings of 279 and 160 Å were found from which a body-centered cubic lattice of side 394 Å and a particle diameter of 340 Å were deduced. The latter value is in reasonably good agreement with the value of 280 Å obtained from sedimentation data. The crystals of bushy stunt virus shrink and swell reversibly on drying and rewetting and the amount of the shrinkage (20 per cent)

was found to be the same whether estimated by direct observation under the microscope or by means of the difference in the lattice dimensions of wet and dry specimens obtained from x-ray photographs. Vinson, McReynolds & Gingrich (175) reported an x-ray diffraction study of tobacco mosaic virus but the spacings which they obtained are in poor agreement with those reported earlier from Wyckoff's and Bernal's laboratories.

Several workers (5, 42, 57, 120) have studied the effect of x-rays, γ -rays and ultraviolet light on purified and unpurified tobacco mosaic virus and in general the survival ratios for the virus followed a simple exponential curve, hence it was concluded that the absorption of one unit of energy by a virus particle is sufficient to cause its inactivation. From such studies, Gowen (41) has calculated that the volume of the tobacco mosaic virus particle necessary for its reproductive activity corresponds to a molecular weight of about 15,000,000. Unfortunately, instead of the experimentally determined value of 1.37, an assumed value of over three was used for the density of the virus in these calculations. The molecular weight based on the correct density becomes 5,600,000. This value should then be compared not with 17,000,000, a molecular weight which was estimated some years ago when the asymmetry of the virus was unknown and which was based on an assumed asymmetry constant of 1.3, but with 50,000,000, a molecular weight value which appears most reasonable in view of several kinds of evidence recently obtained (see page 554). These results would indicate that the volume sensitive to x-rays corresponds to about one ninth of the total volume of the virus molecule. An interesting but unexplained observation is that moderate doses of x- and γ -rays cause an apparent enhancement in the virus activity of solutions of tobacco mosaic virus (57, 66). Kausche & Stubbe (67) have reported briefly some results obtained by irradiating living mosaic diseased leaves with x-rays, which they regard as indicating that the irradiation induced mutation of the virus. Since it is well known that strains of tobacco mosaic continually arise spontaneously during multiplication of the virus in a host, definite proof of induced mutation becomes somewhat difficult although it may be forthcoming with more complete studies.

Size and shape.—Viruses vary in size from the elementary bodies of psittacosis and vaccinia, which are essentially spherical and have diameters of about 275 m μ and 175 m μ , respectively, to the virus of the foot and mouth disease, whose shape is unknown, but which has an effective diameter of about 10 m μ . Intermediate are viruses of

many different sizes and there does not appear to be a definite grouping about any particular size. It should be emphasized, however, that each virus has its own quite definite and characteristic size regardless of its host source. Most of the information concerning the sizes of viruses has come from ultrafiltration studies except in the case of the large viruses where direct observation by means of the ultramicroscope was possible. Ultrafiltration studies do not provide accurate information concerning the third dimension of particles, hence, until recently practically nothing was known about the shapes of the smaller viruses. However, in 1938 it became possible to obtain an image of objects as small as about 10 μ through the use of the electron microscope. Photographs obtained in this manner of vaccine, infectious ectromelia, and myxoma viruses have shown discrete spherical particles of approximately the sizes estimated previously by other methods (22). Perhaps the most interesting of the results obtained with viruses and the electron microscope is that with tobacco mosaic virus (63, 136), for indirect evidence has indicated an unusual shape for this virus. It may be recalled that Takahashi & Rawlins (168) noted some years ago that the juice from tobacco mosaic diseased plants exhibited stream double refraction and concluded that the virus, or something regularly associated with it, consisted of rod-shaped particles. Subsequent work has also indicated that this virus is rod-shaped and, based on viscosity, sedimentation, diffusion, and stream double refraction data, estimates ranging from about 400 to 700 μ for the length and from 11 to 15 μ for the cross section of the particles have been made (35, 76, 81). The propriety of using these data, especially the viscosity data, in this manner has been questioned (31, 32, 33), hence, unusual interest was attached to the photographs of tobacco mosaic virus obtained by means of the electron microscope. These photographs demonstrated not only the existence of discrete particles, but also that these particles are about 15 μ in cross section and about 330 μ in length and hence must have a molecular weight of the order of 50,000,000 (63, 65). The results obtained by indirect means are thus in reasonably good accordance with those obtained by direct observation, hence, the limitations of the indirect methods do not appear to be sufficiently serious to impair their usefulness. This is of importance not only with respect to the theoretical colloid chemistry of asymmetrical particles, but also with respect to viruses for, although none of the viruses as yet isolated affecting animals appear to be asymmetrical, several of the plant viruses do appear to have a

rod-like shape. Loring (90) has, for example, by the use of similar indirect methods estimated that latent mosaic virus is a rod having a length of about 400 m μ , a cross section of about 10 m μ and a molecular weight of about 26,000,000. Cucumber mosaic viruses -3 and -4 (5, 121), potato virus "Y," and Hyoscyamus virus-3 (9) also appear to be very asymmetrical in shape but sufficient data are not yet available to permit an estimation of their dimensions. It may be noted that studies on monolayers of viruses have also contributed to the knowledge regarding their sizes (74, 140).

The purified preparations of several of the smaller viruses do not exhibit double refraction of flow (7, 80, 159) and, since it is likely that these viruses are essentially spherical in shape, their molecular weights and sizes may be estimated directly from sedimentation data. Tobacco ring spot virus has a sedimentation constant¹ of 115×10^{-13} and a specific gravity of 1.57, which, on the basis of a spherical molecule, would correspond to a molecular weight of 3,400,000 and a diameter of 19 m μ (159). The latter value is in agreement with that estimated by ultrafiltration. McFarlane & Kekwick (98) found bushy stunt virus to have a sedimentation constant of 146×10^{-13} and a specific gravity of 1.35 from which a molecular weight of 8,800,000 and a diameter of 27.4 m μ were estimated. The molecular weight calculated from sedimentation equilibrium data was 7,600,000, and the agreement of the two values is an additional indication that the molecule is essentially spherical in shape. Pirie and co-workers (116) found crystalline and amorphous tobacco necrosis virus preparations of the same specific activity to have sedimentation constants of 130×10^{-13} and 58×10^{-13} , respectively, from which molecular weights of 7,400,000 and 2,200,000 were calculated. Neither value is in accord with the sedimentation constant of 112×10^{-13} which was found by Price & Wyckoff (122) for preparations of this virus obtained by differential centrifugation; hence, additional studies will be necessary in order to clarify the apparently puzzling behavior of this virus. Purified preparations of the Shope rabbit papilloma virus have a sedimentation constant of about 250×10^{-13} which on the basis of the usual assumptions would correspond to a molecular weight of 25,000,000 and a particle diameter of about 40 m μ (12).

Pickels & Smadel (115), in a study of the sedimentation behavior of the elementary bodies of vaccinia, found a sedimentation constant of 49×10^{-13} which is in accord with previous results (13) and from

¹ S₂₀. Expressed throughout as cm. sec.⁻¹ dynes⁻¹.

which a particle diameter of 236 m μ was estimated. Smadel, Pickels & Shedlovsky (146) found that when suspended in increasing concentrations of sucrose, glycerol, or urea solutions, the elementary bodies of vaccinia show variations in sedimentation rate which indicate changes in the density or size of the particle. The changes were reversible and were greatest with urea solutions. The view that the elementary body possesses a cell membrane is not shared by McFarlane & Macfarlane (99) who consider that it is more analogous to a protein gel with an ion atmosphere containing much water. Beard, Finkelstein & Wyckoff (14) found the elementary bodies of vaccinia to have a sedimentation constant of 54×10^{-11} and to remain active at hydrogen ion concentrations between pH 5.6 and pH 10.1. Smaller sedimentation constants and inactivation were observed at hydrogen ion concentrations below pH 2.6 and above pH 11.5. Hopwood, Salaman & McFarlane (50) reported that elementary bodies of vaccinia dried in the frozen state could be redispersed by means of ultrasonic radiation with retention of the original activity.

Stern & Duran-Reynals (166) found preparations of the chicken tumor I agent purified by Claude's method (24) to contain a relatively homogeneous component having a sedimentation constant of 550×10^{-12} . They estimated this component to have a molecular weight of 139,000,000 and a particle diameter of 70 m μ . The latter value is in good agreement with those obtained previously by ultrafiltration and nonoptical centrifugation experiments and by means of direct observation with the ultramicroscope (25). Approximately the same values were obtained with purified preparations obtained from the plasma of chickens diseased with fowl leucosis (167). Scott & Elford (139), in ultrafiltration and ultracentrifugation studies based on activity measurements, found the virus of lymphocytic choriomeningitis to have a particle size of between 37 and 60 m μ . Gratia & Paillot (45, 110) have shown that the virus causing jaundice of silkworms can be sedimented in a high speed centrifuge and have estimated the virus to be about 100 m μ in diameter. They consider that particles of this size may aggregate to form the large polyhedral bodies which have been known for some years (38) and have shown that the two forms are related serologically and are different from products obtained from normal tissues.

Kalmanson & Bronfenbrenner (55) obtained a purified *B. coli* bacteriophage preparation by collection on a collodion membrane which retained the phage but allowed smaller extraneous material to

pass, and from activity measurements estimated the phage particles to have a diameter of about 44 m μ and an average molecular weight of about 36,000,000. However, diffusion measurements on the same preparations indicated the presence of active particles ranging in diameter from 4 to 18 m μ and in molecular weight from about 25,000 to about 2,250,000. These preparations contained but 10^{-17} gm. of nitrogen per unit of lytic activity or from five to ten times less than the amounts reported by Northrop (107) and Schlesinger (138), and hence are the most active phage preparations yet obtained. This could be due to the achievement of a greater degree of purity or to the use of a phage of smaller particle size. Diffusion measurements on a staphylococcus phage purified in the same manner indicated the presence of active particles having diameters of from 4.8 to 14 m μ . The range of sizes reported in this work is much lower than that found by Northrop. The difference may be due to the use of different phages, since phages are known to vary widely in size, or it may be due to the fact that Northrop used solutions many times more concentrated than those used by Kalmanson & Bronfenbrenner, for Northrop has shown that in solutions containing about 0.1 mg. of phage protein per cc. most of the particles have a diameter of about 100 m μ , whereas in solutions containing about 0.001 mg. of protein per cc. most of the particles have a diameter of about 10 m μ .

Tobacco mosaic virus.—During the past year there have been investigated certain special properties of tobacco mosaic virus which, although not specific to this virus are, nevertheless, most conveniently studied with it because of its unusual stability and availability. Some of these properties which are due to the unusual shape of the virus have proved of considerable importance in the solution of problems of theoretical chemistry. Langmuir (73) and Levine (88) have shown that it follows from the Debye-Hückel theory that the net effect of the charges on colloidal particles, considered in conjunction with the "gegenions," is an attractive force except when the particles are very near to each other and that the usual procedure of assuming that van der Waals forces are effective over long distances in colloidal systems is unnecessary. Experimental evidence for the existence of long-range forces acting between molecules (16) is provided by the formation of a doubly refracting layer in relatively concentrated solutions of tobacco mosaic virus, and by the demonstration that in this phase and in the wet and dry gels and in the solid crystals the molecules are lined parallel to each other with intervening distances of from about

150 to several hundred Å, depending only upon the concentration. The recognition of these forces is important from the standpoint of the theory of colloidal solutions and may eventually prove of importance in connection with purely biological systems such as those involved in the multiplication of viruses and of cells. Some of the forces which may be involved in these processes have already been discussed (105, 149).

Best (19) has found that the virus concentration range in which the liquid crystalline and isotropic phases can coexist depends upon the electrolyte concentration. In systems at pH 7 and 30° C. the two phases coexist at virus concentrations between 1.4 and 2.2 per cent in the absence of electrolyte, and at concentrations between 1.8 and 2.9 per cent in solutions of sodium chloride more concentrated than 0.005 *M*. In addition to the spontaneous orientation of molecules which occurs in such solutions, the viscosity results of several workers (31, 35, 76, 77), the diffusion results of Neurath & Saum (106), Frampton's (34) excellent photographic record of the elastic nature of certain solutions of virus, and the electrooptical studies of Lauffer (78) indicate that in solutions more concentrated than a few tenths of one per cent there is interaction between particles. Because of these results and the fact that anomalous viscosity can be demonstrated under certain conditions in even very dilute solutions, Frampton (31, 32) has concluded that in all cases the molecules are not dynamically independent, that their solutions do not behave in the manner required by the ideal laws of Stokes, Fick and Poiseuille and hence that the asymmetry and molecular weight values calculated from physical data are wholly ambiguous. However, there appears to be no justification for such a conclusion for the physical data used in the calculations were obtained with solutions containing less than 0.5 per cent virus and there is abundant evidence that the forces of attraction between the molecules at pH 6 to 7 are negligible in such dilute solutions. Lauffer (77) has shown that the viscosity increments at concentrations from 0.01 to 0.1 per cent of virus are directly proportional to the concentration as they should be in the absence of interparticle attraction. The double refraction of flow of solutions of the virus is almost exactly proportional to the virus concentration for even very low velocities of shear and for concentrations up to about 0.5 per cent, hence, in this respect the laws of ideal dilute solutions are obeyed (62, 80, 101). The electrooptical studies on the virus also indicate that in solutions less concentrated than a few tenths of one per

cent the forces of attraction between particles are negligible (78). Also in accord with this conclusion is the fact that in solutions less concentrated than about 0.5 per cent the rates of diffusion and of sedimentation are independent of the concentration of the virus (106, 179). Robinson (131), by measuring simultaneously double refraction of flow, average orientation of particles and resistance to flow, has been able to show that the anomalous viscosity encountered in even very dilute solutions of the virus is due to the change in the average orientation of mutually independent rod-like particles resulting from altering the velocity gradient of flow, rather than to forces of attraction between particles. Finally, the electron microscope photographs (63, 65) show the virus from dilute solutions to be in the form of discrete particles of approximately the size and shape previously estimated by indirect means (81). It may be concluded, therefore, that the forces that have been shown to exist between particles in relatively concentrated solutions of tobacco mosaic virus become negligible in dilute solutions and that no serious errors are involved in the interpretation of physical data obtained with such dilute solutions in terms of particle size and shape.

The electron microscope has been used by Kausche & Ruska (64) in a most interesting study of the reaction between tobacco mosaic virus and colloidal gold particles. The electron micrographs show that in neutral or slightly acidic solutions there is no reaction between the virus and gold particles but that in solutions more acidic than about pH 4.5 the gold particles are individually adsorbed on the virus particles with the formation of a voluminous red precipitate. The gold is protected by the virus under such conditions, for the addition of sodium chloride does not cause the primary red-colored gold particles to aggregate to the blue form as occurs in neutral solution or in the absence of the virus. This is the first case in which the interaction of the two partners in a colloid-chemical reaction has been followed directly by photographic means. Kausche (60) has shown that the gold-sol reaction may be used not only to indicate the presence of tobacco mosaic virus but also to differentiate this virus from the latent mosaic virus.

The structure of the molecules of tobacco mosaic virus and of the aggregates which these form has continued to attract considerable attention. It may be recalled that the solid needle-shaped particles originally obtained by Stanley (154) by the use of salt and acid were regarded as crystals because their size could be varied over a tenfold

range with retention of their characteristic shape, because a mass of the particles showed double refraction, and especially because Wyckoff & Corey (180) obtained an x-ray diffraction pattern characteristic of a crystalline structure. The fibrous aggregates described by Best (19) were also found to show positive double refraction. It may be noted that to date no evidence has been obtained that a single fiber of these aggregates, a single needle-shaped crystal, or a single molecule possesses intrinsic double refraction. Bernal and associates have secured x-ray data which they interpret as indicating that a single molecule of the virus possesses an internal crystalline structure and hence may be regarded as a crystal, but that the needle-shaped aggregates consist of these crystalline rod-shaped molecules arranged laterally in two-dimensional hexagonal close packing (5, 16). No indication was obtained of intermolecular reflections in the other direction, although equipment capable of showing spacings up to 120 m μ was used, hence it was concluded that there was no crystalline intermolecular regularity in the direction of the length. However, the molecules of the virus appear to be about 330 m μ in length, hence the lack of intermolecular regularity in the direction of the length can not be regarded as conclusively proved. Nevertheless, it seems probable that the proposed interpretation of the x-ray data is substantially correct and that it is more definitive to refer to the needle-shaped aggregates as para-crystals. Certain of the conclusions based on x-ray data are substantiated by recent studies by Kausche & Ruska (65) with the electron microscope. The micrographs of aggregates obtained by salting the virus from solution show a fibrous structure with the individual molecules arranged parallel to each other. However, the micrographs provide evidence for no other type of regularity. The middle portions of the needle-shaped aggregates appear more closely packed than the ends, and structures which may be single molecules may be seen protruding at the ends of the needles. The needle-shaped para-crystals have been stained according to the ordinary methods used in bacteriology (57, 61, 69).

Viscosity and double refraction of flow studies by Lauffer (77) indicate that as the isoelectric point of tobacco mosaic virus is approached from either side, there is an aggregation of the molecules, at first end to end aggregation predominating and later side to side aggregation predominating. Best (19) has also secured evidence for linear aggregation for he found the virus to separate from 0.4 *M* solutions of ammonium sulfate at pH 5 in the form of long thin fibers readily visible under the microscope and showing positive double re-

fraction. Although the aggregation of the virus must be largely reversible since the aggregates may be taken into solution readily, the lowered filtrability and increased inhomogeneity in the ultracentrifuge following prolonged exposure to salt solutions indicates that at least a portion of the aggregation is irreversible. A component formed by the irreversible end to end aggregation of two virus molecules is considered to be responsible for a second more rapidly moving boundary which is always found in ultracentrifugal analyses of virus preparations which have been subjected to processes involving harsh treatment (77).

Frampton & Saum (36) and Frampton (31) have reported that solution of tobacco mosaic virus in 6 *M* urea and 0.1 *M* phosphate buffer at pH 7 caused a hundredfold increase in the diffusion constant of the protein and a great reduction in the degree of the anomaly in the viscosity with no marked change in virus activity. The drastic change in the physical state of the protein under the conditions cited has been confirmed by Stanley & Lauffer (163) who found the virus was broken down into low molecular weight protein which was free of nucleic acid, insoluble in water and dilute salt solutions, and showed no double refraction of flow. The velocity of the degradation of the virus was found to vary greatly with the kind and concentration of electrolytes present and with the hydrogen ion concentration. The end result appears to be similar to that encountered in the disintegration of tobacco mosaic virus with sodium dodecyl sulfate (152). Stanley & Lauffer found, however, that in contrast to the reports of Frampton & Saum, the disintegration of the virus was accompanied by disappearance of virus activity. The low molecular weight protein formed by the disintegration of virus was separated from residual virus by differential centrifugation and found to be completely devoid of virus activity. Any virus activity remaining in the urea solutions is associated, therefore, not with the material of low molecular weight, but with the residual virus of high molecular weight. Obviously viscosity data on solutions containing a large proportion of inactive low molecular weight material and a small proportion of virus cannot be interpreted as that of virus. Whether the difference in the activity results is due to the use by Frampton & Saum of a virus preparation containing a large amount of inactive, yet high molecular weight protein which is preferentially disintegrated, or to some more subtle difference must be left to future experimentation.

Price (118, 119) studied the rates of thermal inactivation of the

viruses of tobacco mosaic, tobacco necrosis, alfalfa mosaic, and tobacco ring spot in the untreated infectious juices and found all to follow the course of a first order reaction. Lauffer & Price (79) in a more extended study found the denaturation of purified tobacco mosaic virus in 0.1 M phosphate buffer at pH 7 to proceed as a first order reaction in the range between 67 to 76° C. The specific reaction rate was decreased thirty-five hundredfold by increasing the acidity from pH 7 to pH 5.8. Pfankuch & Kausche (113) reported that tobacco mosaic and latent mosaic viruses are inactivated by phosphatase and consider that the inactivation is due to the enzymatic activity of the phosphatase. However, the results are inconclusive since it was not shown that phosphate had been removed from the virus or that the reaction was one requiring time. The results could be explained equally well on the basis of inhibition of virus activity. Rischkov & Soukhov (128) found that even under optimum conditions tobacco mosaic virus exhibited none of the usual enzymatic activities such as those of oxydase, peroxydase, catalase, protease, asparaginase, urease, amylase, chlorophyllase, and phosphatase.

Sheffield (143) and Bawden & Sheffield (11) in studies on the intracellular inclusions of some virus infected plants compared the properties of the inclusions with those of the purified viruses and showed that insoluble complexes of viruses with protamines and histones, prepared *in vitro*, resembled in many respects the intracellular inclusions. Goldin (39) found that, in the treatment of sections of mosaic-diseased plants with hydrochloric acid, needle crystals of virus were formed in only those cells which previously had contained crystalline inclusions. A direct correlation between cell inclusions and severity of symptoms has also been reported (145), but such correlation does not hold in all cases. Kausche (58, 59) has described the formation *in vitro* of the hexagonal crystals of tobacco mosaic virus which previously had been noted only within living cells. It is possible, however, that the hexagonal crystals may contain material in addition to virus.

Some years ago, Stanley (153) demonstrated that tobacco mosaic virus is not hydrolyzed by trypsin, and since then trypsin digestion has been used by several workers to remove extraneous normal proteins from preparations of this virus. Martin, Balls & McKinney (96) made use of this method in a study of the protein changes in mosaic-diseased tobacco plants. They found that, immediately following inoculation, the virus increases by the displacement of the same amount

or normal protein but that, at a later stage, the diseased plants contain a high concentration of virus in addition to an amount of normal protein comparable in quantity to that found in normal plants. Although Rischkov & Smirnova (127) reported that lowering the amount of nitrogen supplied to mosaic-diseased plants had no effect on the virus activity of the extracted juices, Spencer (150), in better controlled and more extensive experiments, found that the virus activity of the extracted juices was correlated directly with the amount of nitrogen supplied to the plants. The virus concentration in juice from plants receiving an ample nitrogen supply was over eighty times that in juice from nitrogen-deficient plants. Spencer (151) has recently concluded that high nitrogen nutrition increases the rate of multiplication of the virus in the host.

Johnson (53) has reported that certain microorganisms produce a material which inhibits the activity of tobacco mosaic virus. The juices of different insects have been found by Black (20) to have an inhibitory effect on tobacco mosaic and several other viruses. The virus may be separated from the inhibitor, and the virus activity regained, by subjecting the mixture to either ultrafiltration or ultracentrifugation. The inhibitor passes a membrane which retains virus or remains in the supernatant fluid when subjected to centrifugation at a speed which sediments virus. Black regards his experiments as indicating that the chief action of the inhibitor is not upon the virus. The results and conclusions are similar to those of Stanley (153), who several years ago worked with trypsin and other proteins having isoelectric points in the alkaline range. It should be recognized, however, that, although these results provide positive evidence for an effect on the host, they do not preclude the possibility of some direct reaction between inhibitor and tobacco mosaic virus. Although the diffusion constant which Hills & Vinson (47) obtained for tobacco mosaic virus is probably far too large since they did not use sufficient electrolyte to eliminate the accelerating effect of small ions on the virus, their diffusion results with mixtures of trypsin and virus do provide some evidence for a reaction between the two. It seems likely that inhibitors will play an increasingly important role in virus research. Attention is directed to the masking effect encountered in the case of the rabbit papilloma virus in domestic rabbits (68, 144) and to the inhibitor associated with the chicken tumor I virus which Claude (26) considers to be a protein. It is not known whether these inhibitors are serum antibodies or materials of a less specific nature.

Miscellaneous.—Best (18) studied the preservative effect of several reducing systems on the virus of tomato spotted wilt and found that some, cysteine for example, increased the period of retention of virus activity from a few hours to thirty-five days. Stanley (160) found the recovery of plants from the tobacco ring spot disease to be accompanied by a reduction in the virus concentration to about one-sixth that in badly diseased plants, but with no change in the properties of the virus. These results, which were obtained by isolation of the virus by means of differential centrifugation, are in accord with the earlier results of Price which were based on virus activity measurements. Pfankuch (111) has suggested that plant viruses may be detected and differentiated by measuring the turbidity caused by untreated infectious juices in solutions of ammonium sulfate. Köhler (70) has reported that heating the infectious juice of plants diseased with latent mosaic virus near the point of inactivation of the virus results in the formation of less virulent strains. Gratia & Manil (44) have reviewed some of their work on the serological properties of the plant viruses. Green (46), Gortner (40) and Lindegren (89) have contributed to the discussion regarding the origin and nature of viruses. Salaman (137) has reviewed the properties of the different strains of latent mosaic virus. Murayama (104) has described some of the physicochemical properties of broad bean mosaic virus. Kasanis (56) has reported the interesting fact that the cells of severe etch diseased plants contain many apparently crystalline intranuclear inclusions. Hirano & Koyama (48) found epidemic encephalitis virus to be most stable at pH 7.4 and to be unaffected by diastase, but little affected by trypsin and inactivated by lipase. Studies on the action of formaldehyde on chicken tumor I and neurovaccine viruses and a bacteriophage have been reported. All were inactivated by formaldehyde, the phage to a certain extent reversibly (103) and the tumor virus irreversibly (117). In the case of the neurovaccine virus, the inactivation was found reversible in one instance (37) and irreversible in another instance (87). Studies on the increase in phage and gelatinase concentrations in cultures of *Bacillus megatherium* and on the growth of bacteriophage have been described by Northrop (109) and by Ellis & Delbrück (29), respectively. Turner & Fleming (173) and Lennette & Smith (83) studied the maintenance of certain animal viruses in the frozen state and Bauer & Pickels (3) described an apparatus for freezing and drying virus preparations in large quantities under uniform conditions.

Moriyama and associates, in a series of publications which are listed in a review paper (102), have described the isolation of materials referred to as minute-body-forming proteins from the normal and virus-diseased tissues of both plants and animals. They consider that viruses denature the proteins in protoplasm with the production of more virus by the endowment of such protoplasm proteins with a "virus radical." Thus when the virus-induced denaturation has occurred, the minute-body-forming protein which is isolated possesses virus activity, but when isolated from normal "undenatured" protoplasm it possesses no virus activity. This general concept is not in accord with views held by most workers and more convincing proof must be offered before the suggested subtle difference between active and inactive preparations can be accepted. It seems likely that the virus activity is not due to the so-called minute-body-forming protein but to the presence in preparations consisting largely of inactive proteins of viruses possessing the characteristic chemical and physical properties described in earlier sections of this review. The crystallization of vaccine virus has been reported (172) but with no reference to any possible relationship between the crystalline material and the elementary bodies of vaccinia. Until definite proof to the contrary is offered, it must be assumed that here also the activity of the crystalline material is due to the presence of elementary bodies. It should be a relatively simple matter to establish the presence or absence of the elementary bodies and if vaccine virus activity can be demonstrated in their absence it would represent an important advance.

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BIOCHEMISTRY OF THE LOWER FUNGI

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INTRODUCTION

The biochemistry of the fungi has been reviewed on three occasions of this *Review* since its inception: i.e., in Vol. I (1932) by Iwanoff and in Vols. II (1933) and V (1936) by Iwanoff & Zwetkoff. The subject of mycological chemistry is growing at such a rapid rate that the reviewer feels that it would be impossible, even if it were desirable, to attempt to cover the whole field of advances made in the different branches of the subject in the last four years. He also feels that it may be more acceptable to attempt to review one aspect of the subject in which he is particularly interested, namely that of the metabolic products of those fungi which are commonly known as "moulds." When the reviewer first commenced work on this subject over twenty years ago the number of mould metabolic products accurately described was small, and real interest in the subject may be said to date from Carl Wehmer's classical observations (1, 2, 5, 6) that when *Aspergillus niger* van Tieghem is grown on sugar solutions oxalic acid is formed in considerable quantities, and that citric acid is a metabolic product of certain mould species to which Wehmer gave the generic name *Citromyces* (3, 4). Since then a very large number of mould metabolic products, of almost all types known to organic chemistry, have been reported from laboratories in different parts of the world and a record of them has been given by Raistrick and later by Clutterbuck and by Birkinshaw.

There has been a tendency, occasionally expressed to the reviewer, to regard these mould metabolic products as microbiological curiosities, or merely as by-products (whatever that may mean) of no particular interest. That this view is surely a mistaken one appears to be indicated by the very large amounts in which they are often formed. Thus, for instance, kojic acid (5-hydroxy-2-hydroxymethyl- γ -pyrone) has been obtained by May, Moyer, Wells & Herrick from *Aspergillus oryzae* Ahlburg in a yield of 55 per cent of the glucose utilised by the organism; Clutterbuck, Haworth, Raistrick, Smith & Stacey

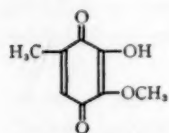
isolated from cultures of *Penicillium Charlesii* G. Smith as much as ten per cent of the glucose metabolised, in the form of carolic acid and its related acids (see pp. 581-583); and the mycelium of *Helminthosporium gramineum* Rabenhorst was shown by Charles, Raistrick, Robinson & Todd to contain at least 30 per cent of its dry weight in the form of a mixture of two polyhydroxyanthraquinones. The reviewer feels therefore that many, if not all, of the mould metabolic products described should be regarded as being of as much potential importance in relation to the species of mould producing them as are any products of microbial metabolism, e.g., lactic acid in the case of the lactic acid bacteria or even ethyl alcohol in the case of yeast.

Many of the mould metabolic products described have now been found to be structurally related to each other and also to other already well-known products of vital metabolism. It is therefore proposed to review some of these interrelationships as fully as possible in the space available.

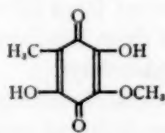
QUINONES

DERIVATIVES OF TOLUQUINONE

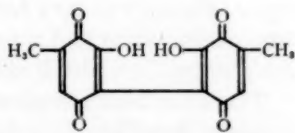
The simplest of the derivatives of toluquinone isolated from mould cultures, and indeed probably the simplest quinone isolated from any natural source is fumigatin, 3-hydroxy-4-methoxy-2,5-toluquinone. This substance which crystallises in maroon coloured needles, m.p. 116°, was reported by Anslow & Raistrick (1) as a metabolic product of an unusual though indubitable strain of *Aspergillus fumigatus* Fresenius and its structure was established without any dubiety by its discoverers as structure I.



I. Fumigatin



II. Spinulosin



III. Phoenicin

Fumigatin is structurally very closely related to spinulosin which was first described by Birkinshaw & Raistrick as a metabolic product of *Penicillium spinulosum* Thom and was shown by them to be a dihydroxy-monomethoxy-toluquinone. Its molecular constitution as 3,6-dihydroxy-4-methoxy-2,5-toluquinone (II) was proved by Ans-

low & Raistrick (1) and was settled by synthesis by the same authors (2). Spinulosin, which is thus 6-hydroxyfumigatin, forms purple-bronze plates, m.p. 201°. Fumigatin was converted by Anslow & Raistrick (1) into spinulosin by a Thiele-Winter acetylation followed by hydrolysis and oxidation of the resulting quinol.

The close relationship between fumigatin and spinulosin thus indicated from the purely chemical point of view is also evident from the observation of Anslow & Raistrick (3) that a different strain of *Aspergillus fumigatus* Fresenius from that which produces fumigatin itself produces spinulosin. These facts would appear to indicate quite different "oxidation levels" in two authentic strains of the same species of mould.

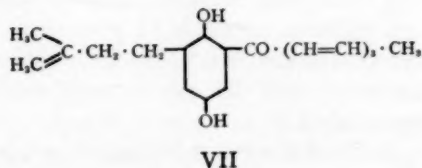
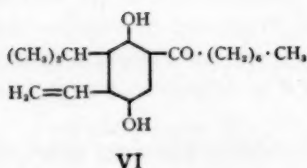
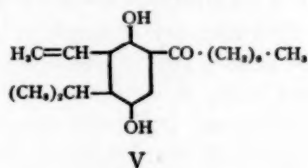
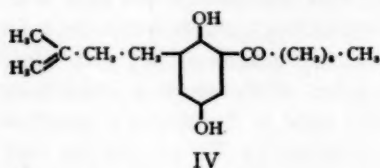
Phoenicin, the red pigment of *Penicillium phoeniceum* van Beyma, forms yellowish brown crystals, m.p. 230–231°. It was first described by Friedheim in 1933 and later by the same worker in 1938. Its constitution as 2,2'-dihydroxy-4,4'-dimethyldiquinone (III) was settled by analytical and synthetic methods by Posternak (1) (who later (2) reported it as a metabolic product of *Penicillium rubrum* Grasberger-Stoll). The close structural relationship between phoenicin on the one hand and fumigatin and spinulosin on the other hand is evident from a comparison of structure III with structures I and II.

The question of the function of these quinones and indeed of a number of other mould metabolic products which will be discussed later is one which has not been settled with any degree of certainty. It is tempting to speculate however, that, because of the ease with which they are reduced to the corresponding quinols which in turn are readily reoxidised to the corresponding quinones, they play some part in the oxidation-reduction mechanism of the moulds which produce them. Indirectly supporting this view is Friedheim's observation (1, 2) that traces of phoenicin increase by 200 to 300 per cent the respiration of unpigmented and washed cells of *Bacillus pyocyaneus*. This fact, together with the actual measurement of the oxidation-reduction potential of phoenicin, led Friedheim to express the view that phoenicin is a thermodynamically reversible oxidation-reduction system and that this property enables it to function as a respiratory catalyst.

Additional support for these views is afforded by the observation of Anslow & Raistrick (1) that there is present in the metabolism solution of *Aspergillus fumigatus* Fresenius, along with fumigatin, the corresponding quinol 3-hydroxy-4-methoxyquinol; also the strictly

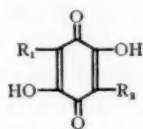
parallel observation of Posternak (2) that, on certain media, particularly beer wort, cultures of *Penicillium rubrum* Grasberger-Stoll remain almost colourless and phoenicin is found in such cultures principally in the form of its leuco derivative, tetrahydrophoenicin. Further, while phoenicin is formed on ordinary Czapek-Dox medium, if the sodium nitrate in this medium is replaced by asparagine or ammonium tartrate there is a preponderance of reduced phoenicin.

A further example of the formation of substituted quinols by moulds (though in this case the corresponding quinones have not been isolated) is afforded by two colouring matters, flavoglaucin and auroglaucin, first reported by Gould & Raistrick as occurring, sometimes in very considerable amounts, in the mycelium of a number of species in the *Aspergillus glaucus* series. Flavoglaucin, $C_{19}H_{28}O_3$ forms lemon-yellow needles, m.p. 105° , and auroglaucin, $C_{19}H_{22}O_3$ orange needles, m.p. 152° . The molecular constitution of flavoglaucin and auroglaucin was investigated by Raistrick, Robinson & Todd and also by Cruickshank, Raistrick & Robinson. The close relationship between the two pigments is shown by the fact that on hydrogenation with palladised strontium carbonate they both give rise to the same product, dihydroflavoglaucin, $C_{19}H_{30}O_3$. While the experimental evidence given by the above authors is not conclusive it is best collated if one of the alternative constitutions (IV), (V) or (VI) is attributed to flavoglaucin. Auroglaucin would then be (VII) on the basis of (IV) or the analogous structures related to (V) and (VI).



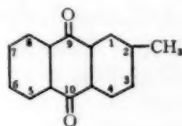
Finally the close relationship of the mould pigment spinulosin to other naturally occurring compounds is shown from a consideration of

the general formula VIII. In spinulosin $R_1 = \text{methyl}$ and $R_2 = \text{methoxy}$. Kögl and his co-workers have shown that in polyporic acid, isolated from the agaric *Polyporus nidulans* Fr., $R_1 = R_2 = \text{phenyl}$ (1) and in atromentin which occurs in the agaric *Paxillus atromentosus* Batch, $R_1 = R_2 = p\text{-hydroxyphenyl}$ (2). In embelic acid (embelin) the active principle of the berries of *Embelia ribes*, Hasan & Stedman showed that $R_1 = \text{H}$ and $R_2 = n\text{-lauryl}$, and Hiramoto considers that in maesaquinone, the orange-red colouring matter in the fruits of *Maesa japonica*, $R_1 = \text{H}$ and $R_2 = \text{C}_{20}\text{H}_{39}$.



VIII

DERIVATIVES OF 2-METHYLANTHRAQUINONE



2-methylanthraquinone

Many of the lower fungi are conspicuous because of their bright colours—yellow, orange, red, purple and violet—and these colours, particularly of the reverse of the colony in culture, are used in their morphological diagnosis. Investigation of the colouring matters with a view to determining their chemical constitution has only been seriously undertaken in recent years but already certain genera of moulds have proved to be very fruitful sources of hitherto undescribed polyhydroxyanthraquinones, all of which have proved, at any rate up to the present, to be derivatives of 2-methylanthraquinone (structure and numbering followed are given above).

From species of Helminthosporium.—One of the most fruitful genera examined has proved to be the genus *Helminthosporium* which includes in its species a large number of well known plant pathogens of considerable economic importance.

The first of these polyhydroxyanthraquinones to be isolated in these laboratories was helminthosporin, which occurs in the mycelium

of *Helminthosporium gramineum* Rabenhorst, the causative agent of "leaf stripe" disease of barley, and together with catenarin (see later) amounted to 30 per cent of the dry weight of the organism grown on a synthetic Czapek-Dox glucose solution. Helminthosporin was shown by Charles, Raistrick, Robinson & Todd to be 4,5,8-trihydroxy-2-methylanthraquinone, and this molecular constitution was confirmed by its synthesis by Raistrick, Robinson & Todd (1). Helminthosporin was also shown to be a metabolic product of other species of *Helminthosporium* notably of *H. cynodontis* Marignoni [Raistrick, Robinson & Todd (2)] which is parasitic on *Cynodon Dactylon* Persoon, an important forage grass in many parts of America and India; of *H. catenarium* Drechsler; and of *H. tritici-vulgaris* Nisikado the causative agent of a yellow spot disease of wheat, a disease of fairly common occurrence in Japan [Raistrick, Robinson & Todd (3)].

On oxidation with manganese dioxide and concentrated sulphuric acid helminthosporin gives 1,4,5,8-tetrahydroxy-2-methylanthraquinone which was shown by Raistrick, Robinson & Todd (2) to be identical with cynodontin. This compound is a mycelial constituent of *H. cynodontis*, of *H. euchlaenae* Zimmermann, a fungus parasitic on the forage cereal known as "Teosinte" in the States on the Gulf of Mexico, and of *H. avenae* Eidam, the causative agent of a leaf spot disease of oats.

Catenarin, the exact molecular constitution of which has not yet been determined, has been shown by Raistrick, Robinson & Todd (3) to be a 1,4,5-trihydroxy-(β -hydroxymethyl)-anthraquinone with the β -hydroxymethyl group either in position 2, 6 or 7. That catenarin is a derivative of 2-methylanthraquinone is shown by the fact that it gives 2-methylanthracene on zinc dust distillation. Catenarin is present along with helminthosporin to the extent of twenty-five per cent of the dry weight of the mycelium of *H. catenarium* and was also isolated from *H. gramineum*, from *H. velutinum* Link and from *H. tritici-vulgaris*.

Tritisporin is believed by Raistrick, Robinson & Todd (3) to be a 1,3,5,8-tetrahydroxy-(β -hydroxymethyl)-anthraquinone with the β -hydroxymethyl group either in position 6 or 7. It gives 2-methylanthracene on zinc dust distillation and was isolated in small amounts from cultures of *H. tritici-vulgaris* Nisikado.

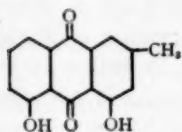
From species of Aspergillus and Penicillium.—It has already been reported (p. 574) that species of fungi in the *Aspergillus glaucus* series, most of which are highly pigmented, have as the major por-

tion of their colouring matters the two substances flavoglaucin and auroglaucin. Many of these species, however, have now been shown to contain, in addition to flavoglaucin and auroglaucin, the long-known anthraquinone derivative obtained from lichens variously known as physcion, parietin and emodin monomethyl ether (4,5-dihydroxy-7-methoxy-2-methylanthraquinone). This substance was isolated from cultures of *Aspergillus glaucus* Link by Raistrick, Robinson & Todd (4) and from each of sixteen other species or strains in the *A. glaucus* series by Ashley, Raistrick & Richards. This observation is of some biological interest since the production of physcion by both a fungus and a lichen (among others by the wall lichen, *Xanthoria parietina*) gives strong evidence for the view that the so-called lichen acids owe their origin to the fungal half of the fungus-alga symbiont.

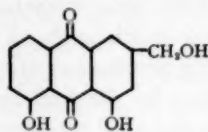
Two other derivatives of frangula-emodin (4,5,7-trihydroxy-2-methylanthraquinone) have just been reported from these laboratories (Anslow, Breen & Raistrick) as metabolic products of a strain of *Penicillium cyclopium* Westling, neither of which has been reported previously from natural sources and one of which has not been previously described. They are the hitherto undescribed ω -hydroxy-emodin [4,5,7-trihydroxy-2-(hydroxymethyl)-anthraquinone] and emodic acid [4,5,7-trihydroxy-anthraquinone-2-carboxylic acid]. Posternak (1939) in a preliminary communication described the isolation from cultures of *Penicillium citreo-roseum* Dierckx of an unnamed colouring matter which had the empirical formula $C_{18}H_{10}O_6$. Posternak believed this compound to be a polyhydroxyquinone. Direct comparison of ω -hydroxyemodin from *P. cyclopium* and the colouring matter obtained by Posternak from *P. citreo-roseum* has shown them to be one and the same substance.

The very close relationship between mould polyhydroxyanthraquinones and those from other widely different natural sources is clearly seen in the structural formulae IX to XVII. Chrysophanic acid (IX) from species of the higher plants *Rheum* and *Rumex*, aloemodin (X) from species of *Aloe*, and rhein (XI) from Chinese rhubarb, bear exactly the same structural relationship to each other as do frangula-emodin (XV, $R = H$) (from *Rhamnus frangula* and a number of other sources), ω -hydroxyemodin (XVI) and emodic acid (XVII) (from *Penicillium cyclopium*). Thus frangula-emodin is 7-hydroxy-chrysophanic acid; ω -hydroxyemodin is 7-hydroxy-aloe-emodin; and emodic acid is 7-hydroxy-rhein. Further helminthosporin (XII) is 8-hydroxy-chrysophanic acid and cynodontin (XIII) is 1,8-

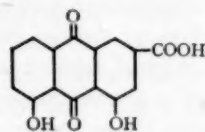
dihydroxy-chrysophanic acid. Finally, although frangula-emodin (XV, $R = H$) has not yet been found in any of the lower fungi it has been isolated by Kögl & Postowsky from the blood-red agaric *Dermocybe sanguinea* Wulf, its 7-methyl ether (XV, $R = CH_3$) occurs as physcion in species in the *Aspergillus glaucus* series and in various lichens, and its 3-carboxylic acid occurs as endocrocin (XIV) in the Japanese lichen, *Nephromopsis endocrocea* Asahina (Asahina & Fuzikawa).



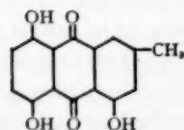
IX. Chrysophanic acid



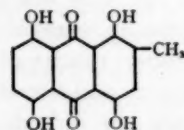
X. Aloe-emodin



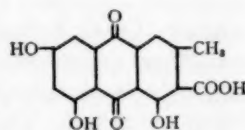
XI. Rhein



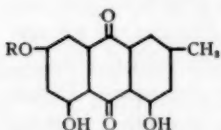
XII. Helminthosporin



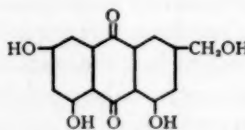
XIII. Cynodontin



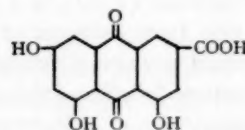
XIV. Endocrocin



XV. Frangula-emodin,
 $R = H$.
Physcion, $R = CH_3$.



XVI. ω-hydroxyemodin



XVII. Emodic acid

Certain other polyhydroxyanthraquinones, the molecular constitutions of which have not yet been established, have recently been isolated from species of moulds. Erythroglaucon, $C_{16}H_{12}O_6$, deep red plates, m.p. $205-206^\circ$, occurs along with physcion in all but one of the sixteen species or strains in the *Aspergillus glaucus* series examined by Ashley, Raistrick & Richards. It is a monomethyl ether of a tetrahydroxymethylanthraquinone, but is apparently not a derivative of cynodontin, as its colour reactions appeared to indicate, since its trimethyl ether is not identical with the tetramethyl ether of cynodontin.

Igarasi reported the isolation from cultures of *Penicillium funiculosum* Thom of a colouring matter, funiculosin, $C_{18}H_{10}O_5$, deep red plates, m.p. 218° , which gives a triacetate and a tribenzoate but contains no methoxy groups. Igarasi believes funiculosin to be either a trihydroxymethylanthraquinone or a (hydroxymethyl)-dihydroxyanthraquinone though this view is difficult to reconcile with his finding that funiculosin gives anthracene and not methylanthracene on zinc dust distillation.

Hind has isolated from cultures of *Penicillium carmino-violaceum* Biourge grown on a glycerol-mineral salts medium two new polyhydroxyanthraquinones, carviolin, $C_{16}H_{12}O_6$, and carviolacin, $C_{20}H_{16}O_7$, both of which are derivatives of 2-methylanthraquinone. Carviolin, chrome yellow crystals, m.p. 286° , is a monomethyl ether and contains three hydroxy groups since it gives a triacetate, a trimethyl ether and a tribenzoate. It is therefore to be regarded as a monomethyl ether of a tetrahydroxy-2-methylanthraquinone or of a trihydroxy-2-(hydroxymethyl)-anthraquinone. Carviolacin, light brown needles, m.p. 243° (decomp.) is also a monomethyl ether, gives a triacetate, a trimethyl ether, and yields 2-methylanthracene on zinc dust distillation.

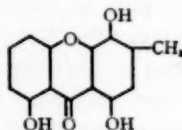
The very frequent occurrence in fungi of polyhydroxyanthraquinones naturally leads to speculation as to their biological function. It must be confessed at once that there is no clear evidence on this point but the reviewer refuses to believe that they can be summarily dismissed as by-products of no biological significance. As with the derivatives of toluquinone, dealt with earlier in this review, one is tempted to regard them as playing a part in an oxidation-reduction mechanism. Two observations seem to support this view. Ashley, Raistrick & Richards found that a few species in the *Aspergillus glaucus* series produce, along with physcion, two reduction products of physcion, i.e., the anthranols, 4,5-dihydroxy-7-methoxy-2-methyl-9-anthranol and 4,5-dihydroxy-7-methoxy-2-methyl-10-anthranol, which, perhaps significantly, were also isolated, as long ago as 1894 by Perkin & Hummel, along with emodin monomethyl ether (physcion) from the root bark of the Indian climbing shrub *Ventilago madraspatana* Gärttn. The second observation arises from an investigation of the metabolic products of *Helminthosporium leersii* Atkinson by Ashley & Raistrick. The mycelium of this species, unlike so many species in this genus which yield polyhydroxyanthraquinones, was found to contain considerable amounts of two metabolic products, luteoleersin $C_{26}H_{18}O_7$, yellow rods, m.p. 135° , and alboleersin $C_{26}H_{40}O_7$, colourless needles,

m.p. 215–216°. These substances are obviously not simple polyhydroxyanthraquinones but they are of interest from the present point of view since they are very readily interconvertible by simple oxidation and reduction processes and Ashley & Raistrick express the view that luteoleersin is a substituted quinone or semiquinone and alboleersin the corresponding quinol.

HYDROXYXANTHONES

Hydroxyxanthones are not of frequent occurrence in nature, the two best known ones being euxanthone (1,7-dihydroxyxanthone), which occurs as the glucoside mangiferin in the leaves of *Mangifera indica* L., the Indian mango tree, and gentisin (1,7-dihydroxy-3-methoxy-xanthone) which is present, probably as a glucoside, in the rhizomes of *Gentiana lutea* L. Two more hydroxyxanthones have recently been described as mould metabolic products.

Ravenelin was isolated by Raistrick, Robinson & White in a yield of about 10 per cent of the weight of the dry mycelium of *Helminthosporium Ravenelii* Curtis and in much smaller amounts from *Helminthosporium turcicum* Passerini, the causative agent of a disease of maize known as "leaf blight." Ravenelin, $C_{14}H_{10}O_5$, gives a triacetate and also a trimethyl ether which forms a ferrichloride, $C_{17}H_{17}O_5 \cdot FeCl_4$, red prisms, m.p. 174–175°. The molecular constitution of ravenelin was shown to be 1,4,8-trihydroxy-3-methylxanthone of structure XVIII.



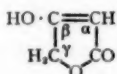
XVIII. Ravenelin

It is of interest to compare the molecular constitution of ravenelin with that of two other *Helminthosporium* colouring matters previously referred to, i.e., helminthosporin (structure XII, p. 578) and cynodontin (structure XIII, p. 578). It will be seen that the methyl group and two of the hydroxyl groups are identically placed in ravenelin and helminthosporin and that the fourth hydroxyl group in cynodontin corresponds to the third of ravenelin.

Rubrofusarin, $C_{14}H_8O_4 \cdot (OCH_3)_2$, was isolated by Ashley, Hobbs

& Raistrick from the mycelium of cultures of *Fusarium culmorum* (W. G. Smith) Sacc. and a number of related forms. Its molecular constitution has not yet been elucidated but it appears to be the mono-methyl ether of a trihydroxymethylxanthone isomeric with but not identical with ravenelin. Rubrofusarin gives a diacetate, and a dimethyl ether which forms a ferrichloride, $C_{17}H_{17}O_5 \cdot FeCl_4$, maroon prisms, m.p. 183–184°.

DERIVATIVES OF TETRONIC ACID



Tetronic acid

A series of mould metabolic products, which may be regarded as derivatives of tetronic acid, has been described in recent years and affords an excellent example of a close structural relationship.

The metabolic products of a new species of *Penicillium* (*Penicillium Charlesii* G. Smith), isolated from mouldy Italian maize, were examined by Clutterbuck, Haworth, Raistrick, Smith & Stacey and led to the isolation, in a total yield of over 10 per cent of the glucose metabolised, of the following substances: *l*-γ-methyltetronic acid, $C_8H_6O_3$; carolic acid, $C_9H_{10}O_4$; carolinic acid, $C_9H_{12}O_7$; carlic acid, $C_{10}H_{10}O_6$; and carlosic acid, $C_{10}H_{12}O_6$.

A clue to the molecular constitution of these acids was afforded by the products obtained by hydrolysis. These acids on boiling with 2*N* sulphuric acid break down quantitatively into a series of simple compounds. Under these conditions *l*-γ-methyltetronic acid gives one molecule of carbon dioxide and one molecule of acetoin; carolic acid gives one molecule each of carbon dioxide, acetoin and butyrolactone; carolinic acid gives one molecule each of carbon dioxide, acetoin and succinic acid [Clutterbuck, Raistrick & Reuter (1)]; carlic acid gives two molecules of carbon dioxide and one molecule each of acetoin and butyrolactone; and carlosic acid gives two molecules of carbon dioxide and one molecule each of acetoin and *n*-butyric acid [Clutterbuck, Raistrick & Reuter (2, 3)]. These, and other considerations for which the original communications must be consulted, led to the following structural formulae being assigned to the acids from *P. Charlesii*: *l*-γ-methyltetronic acid, XIX; carolic acid, as the hydrated form, XX;

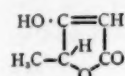
carolinic acid, XXI; carlic acid, as the hydrated form, XXII; and carlosic acid, XXIII.

In 1936 Birkinshaw & Raistrick, in an investigation of the metabolic products of strains in the *Penicillium terrestre* Jensen series isolated a new mould metabolic product, terrestric acid, $C_{11}H_{14}O_4$, which is very similar in its properties to carolic acid. On acid hydrolysis it gives one molecule each of carbon dioxide, acetoin, and the *l*-isomeride of *n*-hexanolactone. From the structural formula (XXIV) assigned to the hydrated form of terrestric acid it is obvious that terrestric acid is to be regarded as ethylcarolic acid.

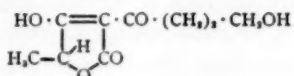
Penicillic acid was first described by Black & Alsberg in 1910 and later by Alsberg & Black in 1913 as a metabolic product of *Penicillium puberulum* Bainier. These workers assigned to it the empirical formula, $C_8H_{10}O_4$, and described its properties and certain of its derivatives but did not suggest a structural formula for it. Penicillic acid was encountered again by Oxford & Raistrick in the metabolic products of a strain of *Penicillium cyclopium* Westling and its molecular constitution was investigated by Birkinshaw, Oxford & Raistrick. Its properties are best illustrated by assigning to it the interchangeable structural formulae XXV and XXVI. Structure XXVI may be rewritten as structure XXVII from which penicillic acid is seen to be the β -methyl ether of γ -hydroxy- γ -isopropylidene tetronic acid.

The close structural relationship of this series of metabolic products to ascorbic acid (vitamin C), structure XXVIII, evident from a comparison of their molecular constitutions, naturally leads one to expect to find vitamin C itself as a mould metabolic product. This expectation is strengthened by the fact that vitamin C is often associated with citric acid in nature and citric acid is a common mould metabolic product. Up to the present no claim has been made for the actual isolation of vitamin C from mould cultures although in a number of recent publications, presumptive, though not by any means conclusive, evidence has been reported. Bernhauer, Gorlich & Kocher report that cultures of *Aspergillus niger* grown on sucrose solutions at pH 2.8–3.0 contain a substance which reduces 2,6-dichlorophenol-indophenol, and iodine in acid solution. Similar observations are made by Fukumoto & Shimomura for a number of species of *Aspergillus* and *Penicillium*. They failed however to demonstrate in the guinea pig any vitamin-C-like action of the concentrated and purified solution of the reducing substance. Finally Manceau, Policard & Ferrand, in a paper which gives a good bibliography of the subject, state that the

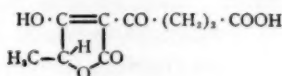
reducing substance formed, on Czapek medium containing mannose or fructose, by cultures of *Penicillium citreo-roseum* Dierckx and *P. ochraceum* Bainier is not ascorbic acid.



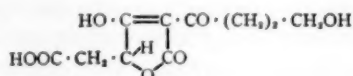
XIX. γ -methyl-tetronic acid



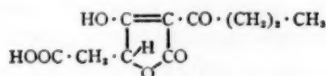
XX. Carolic acid



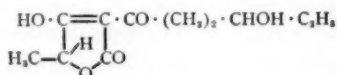
XXI. Carolinic acid



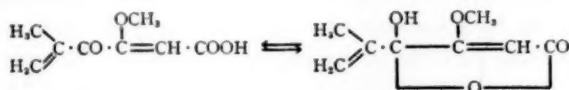
XXII. Carlic acid



XXIII. Carlosic acid



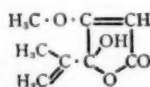
XXIV. Terrestric acid



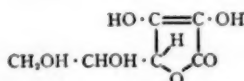
XXV

Penicillic acid

XXVI



XXVII. Penicillic acid



XXVIII. Ascorbic acid (vitamin C)

UNCLASSIFIED MOULD METABOLIC PRODUCTS

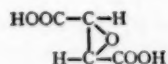
There have been described recently a number of mould metabolic products which do not fit naturally into the scheme of classification adopted in this review. A selection from these is included in this section since it is the writer's opinion that they may ultimately prove to be either important links in the chain of intermediate mould metabolism, or that they will, in due course, be correlated with other better known natural products.

Malonic acid, $\text{HOOC} \cdot \text{CH}_2 \cdot \text{COOH}$, was isolated by Igarasi from cultures, on a synthetic glucose medium, of *Penicillium funiculosum* Thom from which species the same worker also isolated the polyhydroxyanthaquinone, funiculosin (see p. 579).

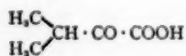
Sakaguchi, Inoue & Tada, working with two new mould species, *Monilia formosa* nov. sp. and *Penicillium viniferum* nov. sp., have obtained, in addition to citric acid, 10 per cent yields of *l*-ethylene oxide- α , β -dicarboxylic acid of structure XXIX, from cultures grown on a synthetic glucose medium. Since this acid is readily converted into *meso*- and *d*-tartaric acids by boiling its aqueous solution, the authors suggest that an industrial process for the manufacture of tartaric acid based on the use of these moulds might be developed.

Hida reported that established cultures of *Aspergillus niger* when transferred to five per cent glucose or sucrose solutions containing five per cent of sodium sulfite produce, in addition to pyruvic acid, dimethylpyruvic acid (XXX), the methyl ester of which was obtained as an oxidation product of dihydropenicillic acid by Birkinshaw, Oxford & Raistrick.

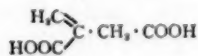
Itaconic acid (XXXI), which is obviously closely related structurally to dimethylpyruvic acid, was first described as a mould metabolic product by Kinoshita, who isolated it in good yield along with citric acid from cultures of *Aspergillus itaconicus*. Itaconic acid has also been reported as a metabolic product of a strain of *Aspergillus terreus* Thom by Calam, Oxford & Raistrick. The latter authors could find no evidence in support of the view, expressed by Kinoshita for *A. itaconicus*, that citric acid is an intermediate stage in the formation of itaconic acid by *A. terreus*.



XXIX. *l*-ethylene oxide- α , β -dicarboxylic acid



XXX. Dimethylpyruvic acid

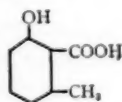


XXXI. Itaconic acid

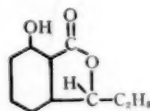
Walker and Bond, Knight & Walker have observed that cultures of *Aspergillus parasiticus* Speare and of a strain of *Aspergillus flavus*, after plasmolysis by toluene, bromobenzene, or chloroform, convert soluble starch, maltose, sucrose, or glucose in dilute aqueous solution into glucosone, $\text{CH}_2\text{OH} \cdot (\text{CHOH})_4 \cdot \text{CO} \cdot \text{CHO}$. The two species named also produce kojic acid, 5-hydroxy-2-hydroxymethyl- γ -pyrone,

during normal growth on a glucose medium and *A. flavus* produces it on a glucosone medium as well. The possibility exists therefore that glucosone is one of the early stages in the conversion of glucose into kojic acid by moulds.

The following new mould metabolic products are of interest as examples of the formation of simple aromatic compounds by fungi. Birkinshaw & Findlay, in the first of a series of investigations on the wood-rotting fungi, find that cultures of *Lentinus lepideus* Fr. give rise to methyl *p*-methoxycinnamate, methyl cinnamate and an ester (probably the methyl ester) of anisic acid. Mellein, $C_{10}H_{10}O_3$, a metabolic product of *Aspergillus melleus* Yukawa was first isolated and described by Nishikawa (1) and was later shown by the same author (2) to give, on fusion with potassium hydroxide, 6-hydroxy-2-methylbenzoic acid (XXXII) which had previously been found as a metabolic product of *Penicillium griseo-fulvum* Dierckx by Anslow & Raistrick. About the same time Yabuta & Sumiki (1) described ochracin, $C_{10}H_{10}O_3$, a metabolic product of *Aspergillus ochraceus* Wilhelm and the same authors (2) identified ochracin as being identical with mellein and suggested that it is a lactone of 6-hydroxy-2-(α -hydroxypropyl)benzoic acid (XXXIII).

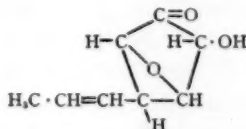


XXXII



XXXIII. Ochracin or mellein

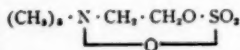
Terrein, $C_8H_{10}O_3$, was isolated by Raistrick & Smith from cultures of two strains belonging to the group species *Aspergillus terreus* Thom, grown on Czapek-Dox five per cent glucose solution. Terrein is a colourless, crystalline, powerfully reducing substance and was shown by Clutterbuck, Raistrick & Reuter to be 4-propenyl-2-hydroxy-3,5-oxidocyclopentane-1-one (XXXIV).



XXXIV. Terrein

Palitantin, $C_{14}H_{22}O_4$, a colourless crystalline metabolic product isolated from cultures of *Penicillium palitans* Westling grown on Raulin-Thom glucose medium was described by Birkinshaw & Raistrick and was shown by them to be an unsaturated dihydroxyaldehyde the molecular constitution of which is being investigated at present.

Two mould metabolic products containing sulphur have recently been reported and in each case the sulphur present originally in the culture medium was in the form of inorganic salts. Ovcharov has demonstrated qualitatively the formation of thiourea by cultures of *Verticillium albo-atrum* and *Botrytis cinerea* grown on Richard's medium with asparagine and ammonium salts as sources of nitrogen. Woolley & Peterson, in one of a series of noteworthy investigations on the chemistry of mould tissue, isolated, for the first time from any natural source, cyclic choline sulphate from the mycelium of *Aspergillus sydowi* Bainier and Sartory grown on a glucose mineral salts medium. Cyclic choline sulphate, $C_5H_{12}O_4NS$ (XXXV), was obtained as colourless crystals which on alkaline hydrolysis gave trimethylamine and on acid hydrolysis split into choline and sulphuric acid. The substance may be regarded therefore as the anhydride of a sulphuric acid ester of choline.



XXXV

CHLORINE METABOLISM OF THE LOWER FUNGI

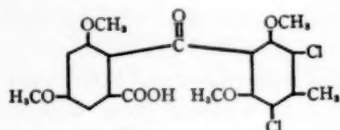
Many of the synthetic media in everyday use for the cultivation of the lower fungi contain chlorides, often of sodium, potassium, calcium, or magnesium. One of the most commonly used of these media is the well known Czapek-Dox solution of the following composition: Glucose, 50 gm.; $NaNO_3$, 2.0 gm.; KH_2PO_4 , 1.0 gm.; KCl , 0.5 gm.; $MgSO_4 \cdot 7H_2O$, 0.5 gm.; $FeSO_4 \cdot 7H_2O$, 0.01 gm.; distilled water, 1 l. The incorporation of chlorides in this type of medium appears to be traditional although there is little, if any, evidence that the chloride ion can be regarded as essential for growth, at any rate in the sense that the elements carbon, nitrogen, phosphorus, and potassium are.

Recently S. L. Mukhopadhyay, working in the reviewer's laboratory, has made a survey of chloride utilisation from the Czapek-Dox medium by a large number of different species of moulds in a number

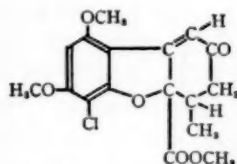
of different genera, the results of which will be published shortly. This investigation has shown that of 139 species or strains examined by far the greater number utilised less than 10 per cent, and only five utilised more than a quarter, of the chloride originally present in the medium. It is safe to say that the entire omission of chlorides from the medium is not detrimental to growth, and in fact neither the original Raulin medium nor the Raulin-Thom modification of this medium (Thom), both of which give luxuriant growth with almost all moulds, contain any chlorides.

Mukhopadhyay's survey arose out of the observation of Raistrick & Smith that a strain of *Aspergillus terreus* Thom when grown on Czapek-Dox medium metabolises over 90 per cent of the chloride originally supplied as potassium chloride, only 6 per cent of which remains as ionisable chloride at the end of the incubation period. The chloride utilised appears in the metabolism solution almost entirely in the form of two new mould metabolic products containing chlorine, i.e., geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_7Cl_2$. The close relationship between geodin and erdin, suggested by their empirical formulae, was confirmed by Clutterbuck, Koerber & Raistrick (1937) who showed that dihydrogeodin and dihydroerdin, the products of the catalytic reduction of geodin and erdin, both yielded the same compound on complete methylation with diazomethane, i.e., a neutral substance having the formula, $C_{15}H_8O_2Cl_2(OCH_3)_5$.

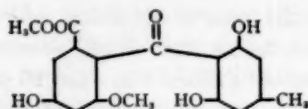
The molecular constitutions of geodin and erdin themselves have not yet been fully elucidated but Calam, Clutterbuck, Oxford & Raistrick have established, by analytical and synthetic methods, the molecular constitution of the trimethyl ethers of dihydrogeodin and dihydroerdin. Dihydrogeodin trimethyl ether was shown to be methyl 3',5'-dichloro-4,6,2',6'-tetramethoxy-4'-methylbenzophenone-2-carboxylate (XXXVI, $COOH = COOCH_3$) and dihydroerdin trimethyl ether to be 3',5'-dichloro-4,6,2',6'-tetramethoxy-4'-methylbenzophenone-2-carboxylic acid (XXXVII). Dihydrogeodin is thus the methyl ester of dihydroerdin.



XXXVI. Dihydroerdin



XXXVII. Griseofulvin



XXXVIII. Sulochrin

Raistrick & Smith (1936) observed that the substitution of potassium bromide or potassium iodide for potassium chloride in equivalent amounts in the Czapek-Dox medium did not result in the metabolism of any appreciable amounts of bromine or iodine by *Aspergillus terreus* although practically normal growth of the mould was obtained in both cases.

The dry mycelium of *Penicillium griseo-fulvum* Dierckx grown on Czapek-Dox solution contains almost 2 per cent of a colourless, crystalline, chlorine-containing metabolic product, griseofulvin $C_{17}H_{17}O_6Cl$, which was described and investigated by Oxford, Raistrick & Simonart. Griseofulvin is strongly dextrorotatory, contains a carbonyl group, a methyl ester group and two methyl ether groups. On oxidation with potassium permanganate it gives, *inter alia*, 3-chloro-2-hydroxy-4,6-dimethoxybenzoic acid. These findings together with other experimental evidence led Oxford, Raistrick & Simonart to suggest structure XXXVII as a tentative formula for griseofulvin.

The above chlorine-containing metabolic products emphasize the close metabolic relationship between moulds and lichens, previously noted in this review, since two of the very few organic chlorine-containing substances occurring in nature have been isolated from lichens, i.e., gangaleoidin, $C_{18}H_{14}O_7Cl_2$, from *Lecanora gangaleoides*, and diploicin, $C_{18}H_{10}O_8Cl_4$, from *Buellia canescens* (Nolan; Hardiman, Keane & Nolan).

Nishikawa (1, 2) extracted from the mycelium of *Oöspora sulphurea-ochracea* van Beyma a number of crystalline substances one of which, sulochrin, $C_{17}H_{16}O_7$ (yellow needles, m.p. 262°), has been shown by the same author (3) to have the probable structural formula, XXXVIII. Although sulochrin does not contain chlorine it is described in this section because of its obvious close relationship to dihydrogeodin and dihydroerodin, the formula for the trimethyl ethers of which is given in structure XXXVI. All three substances are clearly substituted benzophenones. The close relation between these substances becomes even more evident by comparison of their breakdown products with strong sulphuric acid. By this treatment sulochrin breaks down into the monomethyl ether of α -resorcylic acid methyl

ester and *p*-orsellinic acid; dihydrogeodin and dihydroerodin, on similar treatment, give the monomethyl ether of α -resorcylic acid and 2,6-dichloro-3,5-dihydroxy-*p*-toluic acid, i.e., dichloro-*p*-orsellinic acid. Finally, sulochrin is seen to be related to the xanthone, ravenelin, from *Helminthosporium Ravenelii* (XVIII, p. 580), since on treatment of demethylated sulochrin with concentrated sulphuric acid a substituted xanthone is formed which has the methyl group, one hydroxy group, and the carbonyl group in the same relative positions to each other as in ravenelin.

ORGANO-METALLOIDAL METABOLIC PRODUCTS

It has been suspected for a century that cases of arsenical poisoning, ascribed to the use of domestic wall papers pigmented with colours containing arsenic (e.g., Scheele's green) might have their origin in the growth of moulds on the damp wall paper and conversion by moulds of the arsenical colouring matters into volatile and toxic arsenical compounds. The subject was investigated spasmodically by a number of workers (for early history see Challenger) but until 1891, when Gosio began a systematic study of the whole question, little reliable information was available. Gosio (1, 2, 3) found that *Penicillium brevicaulis* Saccardo [*Scopulariopsis brevicaulis* (Sacc.) Bainier] grown on potato mash to which inorganic arsenic compounds had been added gave off a gas with an intense odour of garlic which he believed to be diethyl arsine, $(C_2H_5)_2 \cdot As \cdot H$.

The subject was reopened in 1931 by Challenger and his colleagues at Leeds and was later extended by them to elements other than arsenic, i.e., selenium and tellurium, strains of *P. brevicaulis* being used in all experiments unless otherwise stated. Challenger, Higginbottom & Ellis established the fact that cultures grown on sterile moist bread gave trimethyl arsine, $(CH_3)_3As$ from arsenious oxide, sodium methyl arsonate, and sodium cacodylate, and dimethylethyl arsine, $(CH_3)_2 \cdot C_2H_5 \cdot As$, from sodium ethyl arsonate. Challenger & Ellis extended these observations and obtained methyldiethyl arsine from diethylarsinic acid, dimethyl-*n*-propyl arsine from *n*-propylarsonic acid, and dimethylallyl arsine from allylarsonic acid. No evidence of the formation of alkylated antimony compounds from potassium antimonyl tartrate could be obtained. Other mixed alkylated arsines were isolated by Challenger & Rawlings, all of which contained at least one methyl group. Conclusive evidence of the formation of dimethyl selenide, $(CH_3)_2Se$, from cultures containing sodium selenite or sele-

nate was afforded by Challenger & North, and of dimethyl telluride, $(\text{CH}_3)_2\text{Te}$, from cultures containing potassium tellurite by Bird & Challenger. The same authors also observed the formation of dimethyl selenide and dimethyl telluride with strains of *Penicillium notatum* Westling and *P. chrysogenum* Thom and suggested that the methylation of the metalloids arsenic, selenium, and tellurium is a widespread phenomenon although its mechanism is somewhat obscure (Challenger & Higginbottom).

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THE APPLICATION OF MICROCHEMISTRY TO BIOCHEMICAL ANALYSIS

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The general field of quantitative microchemistry as applied to biochemical analysis, reviewed in 1937 (1), will be revised briefly in the light of the more important contributions made to the literature since that time. In addition, certain other phases of microchemistry not previously considered will be treated in this review. It is still impossible to discuss all of the microchemical methods and techniques that have found a sphere of usefulness in the field of biochemical analysis, due to their large number and wide diversity. Notable among recent trends is the tendency to analyze for smaller amounts of material than was considered possible even a few years ago, and to employ more frequently for analytical purposes a variety of physical instruments including photometers of several types, photoelectric colorimeters, spectroscopes, polarographs, and other devices.

INORGANIC CONSTITUENTS

Magnesium analysis in biological materials has been advanced by the use of ammonium hexaperchloratocerate to oxidize the hydroxyquinoline in the precipitate of magnesium hydroxyquinolate (2). This procedure is superior to the bromination method due to the absence of loss of reagent during the reaction, and the favorable factor of 59.7 equivalents of cerate per mol of magnesium. Elving & Caley (3) have described an oxalate precipitation for this element in glacial acetic acid which provides a simple and accurate method for small amounts.

Sobel & Sklersky (4) devised an ingenious acidimetric micro-method for the analysis of blood calcium, which avoids the use of unstable reagents. Its range and accuracy are about the same as other standard methods. A useful summary and evaluation of twenty-seven micro- and ultramicromethods for calcium and forty-seven micro- and ultramicroprocedures for phosphorus in biological materials was published by Manly (5). It has long been known but not generally recognized that the colorimetric determination of phosphorus based

on the reduction of phosphomolybdic acid is not only empirical but frequently unreliable, i.e., different reducing agents give different colors; acidity, temperature, and concentration of reagents influence the color formation; and little is known of the chemistry of the reaction except that several phosphomolybdates exist, and that molybdic acid itself can be partially reduced. To the solution of these problems, Berenblum & Chain (6) have made a very fundamental contribution by carefully studying these factors and others and minimizing or eliminating them (7). Okamura (8) has avoided some of the difficulties in colorimetric phosphate determination in 0.2 ml. of blood by a preliminary precipitation and separation of the phosphate with cerous chloride. Lundsteen & Vermehren (9) reduced the size of blood sample for phosphorous analysis to 50 μ l. on which duplicate analyses were made with a step photometer with microcell.

The analysis of biological materials for iron has undergone a basic improvement in the introduction by Saywell & Cunningham (10) of *o*-phenanthroline as a colorimetric reagent of very high sensitivity and considerable specificity for this element. The method was further refined by Fortune & Millon (11), and is to be considered equal if not superior to any other colorimetric method for iron so far described.

The use of dithizone as a reagent for various of the heavy metals, particularly lead and zinc, has increased and been profitably extended into the fields of toxicology, spray residue analysis, nutritional studies, and many other related activities. A fairly comprehensive discussion and bibliography of the use of the dithizone system of analysis for heavy metals is given by Wichmann (12). Hibbard (13) applied dithizone to zinc analysis in studying the relation of zinc to the nutrition of plants (cf. 201).

Chloride analysis in biological materials has received much recent attention. Sendroy published a very extended study of the use of solid silver iodate, a method originally announced by Haslewood & King (14). From this study has emerged a gasometric (15), a volumetric (16), and a colorimetric (17) method as well as supplementary publications (18). Saifer & Hughes (19), Weckel (20), and others have extended the use of dichlorofluorescein as an adsorption indicator for direct titration of chloride. Lang (21) has revived and improved the mercurimetric method for blood chloride. Keys (22) modified the Volhard titration for use with 0.2 ml. of blood or other physiological fluid. It remains questionable in the author's opinion

whether there is still a simple, thoroughly dependable method for small amounts of chloride in any and all types of biological materials.

Analysis for biological iodine has progressed chiefly through the development of modifications and some improvements in the older methods. Matthews, Curtis & Brode (23) have further improved the Leipert chromic acid method. An interesting variation is that of Jong (24) who destroyed organic matter with permanganate and eventually separated his iodine as silver iodide instead of employing the usual distillation. McClendon *et al.* (25) have described an elaborate but delicate dry combustion method applicable to 0.2 μ g. of iodine in blood and other materials. Leipert (26) has recently reviewed the subject of iodine determination.

The analysis for arsenic in biological materials has long been one of the least satisfactory from the standpoint of precision and accuracy. This is no doubt due to the very low concentration in which this element is usually found and the fact that the simplicity and great sensitivity of the classical Gutzeit method have appealed strongly to analysts. The sources of error in this method and others involving production of arsine have been partially studied by Mühlsteph (27), Rosenfels (28), Diemair & Fox (29), Cassil (30), and Davis & Maltby (31). Recent highly standardized modifications of the Gutzeit method have been given by How (32), Lockwood (33), and Griffon & Thuret (34). Morris & Calvery (35) separated arsine but determined the arsenic colorimetrically as arsenomolybdate. To the known errors of arsine generation, this would seem to add those of the colorimetric procedure which is similar to that for phosphate and probably contains similar errors (see discussion of phosphorus). Allcroft & Green (36) likewise liberated arsine but titrated the arsenic in the form of arsenious acid, using iodine. Cassil & Wichmann (37) have recently developed a very promising volumetric method for from 5 to 500 μ g. of arsenic. A discussion of the more advantageous recent methods is given by Wichmann (38). Multiplicity of methods, as in this case, may usually be interpreted as a definite indication of basic difficulties. Constituents for which thoroughly satisfactory methods exist receive little attention from analytical investigators.

ORGANIC CONSTITUENTS

So many new methods for organic constituents of biological interest have appeared that it is impossible to do more than mention some of the more significant trends and individual developments. Notable

among trends is a greater use of enzymes in analytical procedures. While the accuracy and precision are frequently not improved by their use, the specificity of the reaction is increased and more confidence is gained in the result. Blauch & Koch (39) used uricase for the determination of uric acid in blood without markedly improving precision over that of the older methods which were unquestionably not specific. Specificity is even more important in the analysis of creatine and creatinine for which Miller and co-workers (40) have developed an enzymic method. Hassid, MacCready & Rosenfels (41) have effectively applied the amylase of saliva to the hydrolysis and subsequent analysis of starch in plant material. Lehmann (42) likewise applied enzymes to the analysis of lactic acid. Miller & Muntz (43) developed for this constituent a delicate colorimetric procedure, while Gordon & Quastel (44) achieved a notable improvement in the usual oxidation method for lactic acid by use of ceric sulfate instead of permanganate, thus rendering unnecessary the removal of sugars prior to the determination.

Numerous improvements in the micromethods for analysis of amino acids and amino nitrogen have appeared. Haarmann (45) and Pope & Stevens (46) developed copper methods modified from that of Kober. The method of Pope & Stevens has been used very successfully in the author's laboratory. Van Slyke & Dillon (47) and Mason (48) described highly specific gasometric procedures for amino acids in which the carbon dioxide released by decarboxylation with ninhydrin was measured. Schlayer (49) modified the method for use with the Warburg apparatus. Virtanen & Laine (50) developed a step-photometric ninhydrin method. Bergmann & Stein described the application of a solubility product method for determining individual amino acids in a mixture (51) to the analysis of collagen and gelatin (52). Toennies & Callan (53) published a careful study of the glacial-acetic-perchloric acid titration of Harris, a method which shows promise of being useful for amino acid determination.

GAS ANALYSIS

Many gas-analysis methods for use with biological materials have been developed and utilized for limited and specific problems, e.g., the determination of ethylene in fruits (54), carbon monoxide in air (55), or the determination of gaseous anaesthetics in blood (56). Systematic microanalytical procedures for gases have not been numer-

ous, though a few quite satisfactory and widely useful systems are now available for biological microanalysis. The simplest and one of the most widely applicable techniques is that originally described by Blacet & Leighton (57), which has been amplified and extended to include water vapor, carbon dioxide, oxygen, hydrogen, carbon monoxide, hydrogen chloride, ammonia, nitrous oxide, methane, and other gases (58, 59, 60). The usual initial sample volume was about 0.1 ml. and the accuracy was usually within a few tenths of 1 per cent. The apparatus was modified, retaining the same range and general technique, by Swearingen, Gerbes & Ellis (61), who eliminated some of the difficulties of construction and some of the previously necessary corrections. Prescott & Morrison (62) have described a complicated but highly refined apparatus with which they have been able to analyze 5 to 25 μ l. of a gas mixture for water, carbon dioxide, hydrogen, carbon monoxide, oxygen, and methane, with an accuracy for each constituent of 2 per cent of the total sample volume. This appears to be the smallest quantity of gas that has so far been systematically analyzed, and reaches the lower limit of volume likely to be encountered in most problems of the biochemist. Clarke & Hermance (63) have described an interesting dilatometric technique for microanalysis of gases. Gilmour (64) developed a dry absorption method for analysis of small amounts of carbon dioxide and oxygen. The most recent review on the general subject of gas analysis, as applied to biological problems, is that of Schwarz & Rappaport (65). This publication did not include the developments listed in this section.

Microrespirometry.—The phase of gas analysis most generally important to biochemists, viz., respirometry, has received much attention. The most widely used and universally applicable instrument has been the well-known Warburg manometric apparatus. Since it is capable of detecting volume changes of only about 0.5 μ l., it will not be considered here as a microinstrument in comparison with the many more delicate types that have been described. Laser (66) and Meier (67) used reduced scale instruments similar to that of Warburg, sensitive to about 0.1 μ l., while Bodine & Orr (68) modified the Krogh (69) instrument to obtain a sensitivity of about 0.02 μ l. These instruments were used in measurement of respiration of tissue cultures, of *Drosophila melanogaster* and similar fairly large systems. Fenn (70) increased the sensitivity greatly (to 0.001 μ l.) with a symmetrical differential instrument with which he could determine both the oxygen consumed and the carbon dioxide produced. This instrument

was modified for still higher sensitivity for the study of stimulated nerve by Schmitt (71). The latter instrument required a very careful control of temperature. Asymmetrical differential respirometers have been constructed by Duryee (72) and by Victor (73) with lower sensitivities (about 0.1 and 0.01 μ l. respectively). Stefanelli (74) used an instrument somewhat similar to that of Victor, with a sensitivity of 0.003 μ l., for measuring the effect of cleavage on the respiration of a single egg of *Rana fusca*. Approximately the same sensitivity was achieved by Heatley, Berenblum & Chain (75), using the principle of an aneroid barometer with exterior pressure compensation and an optical lever for magnification. This instrument permitted the mixing of solutions within the chamber and introduction of any desired gas mixture, features not found in the designs already discussed. Cunningham & Kirk (76) have designed and used a symmetrical differential apparatus having the features mentioned and, in addition, adjustable chamber size and sensitivity down to about 0.002 μ l. This instrument requires no thermostat and may be used with a very wide variety of respiring systems.

The use of the Cartesian-diver method of Linderstrøm-Lang (77, 78, 79) for microrespirometry is discussed elsewhere in this review. The sensitivity of this instrument was about 0.002 μ l. It should be noted that the comparison of the sensitivity of this instrument using a water manometer with others using a different type of index has not always been exact (80), since allowance was not made for the fact that the water manometer was readable to about 2 mm., while the index droplet used, e.g., by Schmitt (71), could be read to better than 0.01 mm.

The most sensitive respirometer described is the single capillary type of Kalmus (81) with a sensitivity of at least 0.0005 μ l. Howland & Bernstein (82) used a modification for measuring single cell respiration, while Gerard & Hartline (83) applied it to nerve metabolism, and Waddington, Needham & Brachet (84) to portions of the amphibian embryo. Cunningham & Kirk (unpublished data) constructed a similar but improved capillary respirometer with a sensitivity of 0.00001 μ l. which was used to measure the respiratory rate of single cells of *Paramecium caudatum*, and of single eggs of *Artemia salina*. Barometric pressure and temperature changes necessarily had a great effect on so delicate an instrument, and the time of temperature equilibration (about 1.5 hours) made it somewhat inconvenient to use. The importance of the time lag will depend on the problem at

hand. The large number of types of microrespirometer which have appeared indicates the absence to date of any universally applicable type, a greatly-to-be-desired development for purposes of cross checking and standardization by different workers.

ULTRAMICROMETHODS

Histochemistry.—The study of enzyme action and the distribution of enzymes in an extensive miscellany of animal and plant materials has progressed notably, chiefly through the work of Linderstrøm-Lang and his associates and others who used his ultramicrotechniques. In addition to the dilatometer (85) and Cartesian-diver (86) methods (see p. 609) for measuring enzymic reaction rates, a method for assay of cholinesterase has been described by Glick (87) and applied to the study of the distribution of this enzyme in the gastric mucosa (88) and in the superior cervical ganglia (89). A method for peptidase and catalase determination has been described by Linderstrøm-Lang (90). The distribution of amylase in the barley grain has been studied by Linderstrøm-Lang & Engel (91), using a previously developed method (92). Peptidase studies have been made in the amoeba by Holter & Kopac (93) and in the developing sea urchin egg by Holter, Lanz & Linderstrøm-Lang (94). Doyle (95) applied the histochemical technique to the study of peptidase and catalase activity in certain marine ova, while Holter & Doyle (96) similarly found and studied peptidase and amylase in the extracts of amoeba and paramecia, and Doyle (97) localized the amylase in amoeba. The references cited represent typical investigations in which the histochemical techniques have proved of value rather than complete summaries of the very wide uses to which these methods have been applied. Reviews of this subject have been published by Linderstrøm-Lang (98) and by Glick (99).

Drop analysis.—Ultramicrochemical methods have been advanced both in regard to the number of constituents determinable and particularly in their application to a greater variety of problems. Norberg (100) applied the Linderstrøm-Lang technique to the determination of less than 4 $\mu\text{g.}$ of potassium with satisfactory accuracy. This is less than has been analyzed by any other chemical procedure yet described for potassium. Linder & Kirk (101) used the drop-analysis technique described by Kirk (102) to analyze for amounts of sodium as small as 0.1 $\mu\text{g.}$ with a mean error of about 5 per cent, the error diminishing to a few tenths of a per cent when amounts of 1 $\mu\text{g.}$ or more were determined. The method was applied to the analysis of

minute amounts of blood. Drop-scale methods for the determination of calcium, first described by Lindner & Kirk (103), for amounts as small as 0.5 μg . have been developed and used by Ellis (104) with samples containing about 7 μg .; by Sobel & Sobel (105) for amounts of about 10 μg . in blood serum; and by Van Bergen & Hill (106), also for this quantity. These methods of calcium analysis are finding their chief application in such fields as the study of dental decay, the physiology of small invertebrates, and, to some extent, in the clinic, particularly for pediatric research.

In addition to the older electrometric drop-scale methods for determination of chloride, Wigglesworth (107) described a modified Volhard titration method for use with as little as 0.3 μl . of physiological fluid, for application in studies of insect physiology. The technique was somewhat involved, though it required virtually no apparatus that could not be readily constructed as needed. The error was greater than usual with drop-analysis methods, viz., from ± 5 to 10 per cent. Accurate evaluation of the error from the small amount of data quoted was impossible. McClendon *et al.* (25) have developed a method for amounts of iodine down to 0.2 μg ., which is well within the range of drop-scale work. They did not use the conventional technique, however, and were obliged to start with large samples. Though the desirability of including their work in this section is doubtful, the range and accuracy of the analyses would seem to be those of the usual drop-scale methods. Lindner & Kirk (108) have developed an ultramicromethod for determining phosphorus by titration with a tested range of 0.05 to 8 μg . and an error of not more than 0.5 per cent, except with the smallest samples. This was applied to blood analysis.

A new ultramicroprocedure for the estimation of total nitrogen by the Kjeldahl method was developed by Needham & Boell (109). In comparison with older methods it offers the advantage of avoiding one transfer of digest, an operation requiring perhaps two minutes, by combining in one vessel the digestion and the diffusion. In so doing, the time of diffusion is increased from two hours or less to at least ten and preferably eighteen hours. Their recovery of 97.5 ± 5 to 7 per cent on samples of from 0.5 to 7 μg . of nitrogen does not compare favorably with the 1 to 2 per cent errors experienced with some of the older procedures. Borsook & Dubnoff (110) recently developed a systematic drop-scale analytical system for the nitrogen fractions, total nitrogen, ammonia, amino nitrogen, amides, peptides,

adenylic acid, and nitrates, and applied it to the study of the metabolism of plant seedlings, growing pea embryos, marine invertebrate embryos, and thin slices of mammalian tissues. Their technique is partially that of Linderstrøm-Lang, but includes a number of important innovations. All methods were titrimetric, the endpoints being determined by use of a glass microelectrode. Sample and diffusion vessels were lathe-cut from lucite rod, an advantageous procedure for various reasons. The range of nitrogen found to be determinable was from 0.3 to 10 μg . Though no data were quoted for evaluation of errors, the maximum error was claimed not to exceed ± 2 per cent with better results on the larger samples and in some determinations. Weinstock *et al.* (111) made use of a drop-scale formol titration method for determining the yield of β -alanine from hydrolysis of pantothenic acid. The samples were much larger than those used with the glass electrode titration method. Blood glucose in amounts of 2 μg . or more has been analyzed by Heck, Brown & Kirk (112) with an error not greater than about ± 1 per cent by a cerimetric-ferrocyanide method. A modified Somogyi method has been described for the determination of blood glucose in the range from 2.5 to 30 μg ., and also a method for determination of 0.5 to 5 μg . of allantoic acid (113). Ross & Tonks (114) applied the method of Rappaport & Pistiner (115) to the study of glucose tolerance. An oxidimetric method has been developed for estimation of amounts of lactic acid in the range from 1 to 30 μg ., with an average recovery of 98.4 per cent (116). Average errors on all but the smallest quantities were about ± 3 per cent. The method was used in the analysis of blood from various normal and diabetic humans and from certain animals. A description (43) has been given of a sensitive colorimetric analytical method for lactic acid with an optimum range of 2 to 10 μg . The method for the determination of a few micrograms of succinic acid, described by Thunberg (117), may be considered as an ultramicromethod, though it is based on the discoloration of methylene blue by succinic dehydrogenase, and makes use of none of the usual techniques of quantitative drop analysis.

PHYSICAL MICROMETHODS

Microchemical methods of analysis based on the use of special physical instruments are in many cases advantageous alternatives to the chemical procedures. As a general rule, physical methods are preferred to chemical methods (*a*) when in a particular range, e.g., with

minute traces, they yield more accurate results or yield satisfactory results more easily or more rapidly; and (b) when they yield better results with a particular type of material, sample, or constituent than can be obtained with chemical methods. The usual objections to the general use of special physical instruments are that they are frequently costly, that they are limited to a few special applications, and that they ordinarily require considerable direct experience and frequent practice on the part of the operator. Generally they should be considered to supplement rather than to replace chemical methods. There are, of course, several kinds of determinations which can only be done by physical methods.

Spectrographic analysis.—Emission spectrography has been widely used for quantitative microanalysis of biological materials, chiefly for the trace elements. This technique is necessarily limited to the analysis for elements (chiefly metallic) and is of particular advantage in those cases (heavy metals, etc.) in which the element yields a clear unmasked spectrum without the necessity of troublesome chemical separations as a prelude to the actual generation of the spectrum. Its use for quantitative procedures is vastly more difficult than for qualitative purposes. However, within the last few years, the quantitative applications have been developed until now it is common to analyze for metals, even in traces, to an accuracy of ± 5 per cent, and in a few instances, through series determinations, to reduce the error to as little as ± 2 per cent. An important general application is the simultaneous determination of more than one element in a mixture from the same spectrum. This is particularly advantageous when the elements are chemically similar, e.g., potassium and rubidium (118), which may make a chemical separation very difficult. A limitation of importance is the concentration range which may be used directly. If the element in question is in high concentration, most spectroscopic methods require a preliminary dilution to bring the intensity of the spectral lines within the range necessary for quantitative estimation. Probably the most important limitations to quantitative spectrography are the necessity of considerable experience in the technique and the availability of correctly designed and constructed instruments, most of which are quite costly.

Owens (119) has reviewed briefly the applications of the spectrograph to trace metal analysis in biological and other material. Cholak & Story (120) applied a highly developed technique to the study of lead, tin, aluminum, copper, and silver in biological materials. The advan-

tages of spectroscopic methods in the study of nutrition of the trace elements for which chemical methods may not be convenient were illustrated by the work of Scoular (121) who investigated the nutritional balances of zinc and aluminum in human subjects. Questions concerning the normal and toxic concentrations of metallic poisons may be readily investigated by this procedure as demonstrated by the studies on lead and other toxic elements (cf. 120; 122, 123). Investigation of localized concentrations of elements made possible by the high sensitivity of the method is illustrated by investigations on the tissues of teeth (124), and the distribution of heavy metals in various organs, such as liver, testicle, spleen, kidney, and brain (125).

The great bulk of literature on the biological applications of emission spectrography is well summarized in the books by Brode (126) and by Gerlach & Gerlach (125, cf. 127) and the summaries of the *Summer Conferences on Spectroscopy and Its Applications*, V, VI, and VII (128).

Absorption spectrography and spectrophotometry as special phases of colorimetry, including its extension to the ultraviolet and infrared ranges of the spectrum, serve as extremely useful but relatively undeveloped phases of microanalysis. The usual applications have been to the study of structure of molecules and to various physical and chemical properties of compounds such as tautomerism, reaction rates, etc., rather than to direct quantitative analysis. The use of the spectrophotometer as an adjunct to ordinary colorimetry (i.e., through use of color-forming reagents) has served much more widely than the direct spectrography of unaltered compounds. Spectrophotometric evaluation for purposes of analysis, of compounds either naturally colored or treated to give color, is unquestionably the nearest approach to absolute colorimetry, since monochromatic light may be used, and the absorption of the compound in question may be studied independently of that of any other absorbing material present. This common use of the spectrophotometer has been well discussed by Ashley (129) and by Mellon (130). The invariable property of elements and compounds of showing some absorption in the visible or invisible regions of the spectrum would seem to make absorption spectrography a possible solution to nearly any analytical problem. That this is frequently not attainable is due to a combination of technical difficulties and to the fact that the absorption is due to chromophor groups or radicals within the molecule rather than to the molecule as a whole. Also, preliminary isolation of the material being

analyzed in a pure or nearly pure form is usually necessary because of the varied absorptions of all the impurities and of the solvent. Glass, quartz, and air may also absorb in the same range as that of the compound in question. Absorption spectrography has proved to be a very useful method of analysis of many compounds which show strong and characteristic absorption in measurable regions. Such analyses are characterized by a relatively high sensitivity, an accuracy to a few per cent and an absolute specificity. Vitamins A (131), B₁ (132), B₂ (133), C (134), D (135), and E (136) all show characteristic absorption in the ultraviolet which has been used as an aid in their isolation and purification. Vitamin A (137) and vitamin D (138) have been quantitatively assayed in this manner. This technique has been utilized in analysis of vitamins and hormones (139), in the analysis of cytochrome-*c* (140), in the analysis of brucine and strychnine in mixtures (141), in the analysis of cocaine in cerebrospinal fluid (142) and brain (unpublished data), and in the determination of the oxygen saturation of whole blood (143). A comprehensive summary of the literature of this subject is impossible, the above serving solely as illustrative references. General treatises on the subject of quantitative use of absorption spectrography are given by Mohler (144), Morton (137), Twyman & Allsopp (145), and others.¹

Photometry.—There has been in the past few years a striking increase in the use of a variety of photoelectric colorimeters, all of which are actually photoelectric photometers. Many of the applications of this useful type of instrument have been made without benefit of any great insight into the principles, limitations, or possibilities involved. Some users of the instrument seem to be unaware that the photoelectric cell is not uniformly more sensitive than the human eye, that it cannot correct as does the eye for colors that are off-tone, and that the instrument is dependable only to the extent that the light transmitted by the filter used corresponds to that absorbed by the colored complex being measured and not to that absorbed by interfering impurities. When this correspondence becomes perfect, the instrument is essentially equal in reliability to the spectrophotometer, provided the photoelectric cell and its attendant measuring device are properly designed and operated to give a linear response to light

¹ The most recent publications which have not been available for examination are by Walker (202) and Miller (203).

intensity. Some instruments which have been described are quite adequate, though a good many of the marketed instruments leave much to be desired. In general, it appears that one of the most common deficiencies in the latter is in the matter of satisfactory filters to assure an adequate selection of light for a wide range of uses. A variety of as nearly monochromatic filters as it is possible to make, or better a monochromator, are essential to the realization of the best results. Few, if any, manufacturers offer such equipment. The great advantage of the photoelectric colorimeter is the absence of subjective effects, either inherent or due to fatigue. In addition, greater sensitivity than that of the eye is available with some wave lengths of light measured. An excellent general discussion of this question with an extensive bibliography is to be found in the article by Müller (146). Other recent discussions are given by Aten (147) and Summerson (148). An indefinite number of specific applications have been described, a few representative ones following: for determination of sodium in serum (149); potassium in serum (150); magnesium in serum (151); manganese in biological materials (152); copper (153); iron and copper in biological materials (154); phosphate (155); chloride in biological fluid (156); blood glucose, blood cholesterol, serum phosphorus, plasma proteins, and urine sugar (157); globulin and albumin in blood serum (158); hemoglobin (159); methemoglobin in blood (160); blood iron and hemoglobin (161); uric acid (162); and vitamin A (163). The list of applications is eventually extensible to include the entire range of colorimetric analysis. It is to be hoped that the attractiveness of this instrument will not cause investigators and analysts to lose sight of the fact that, regardless of how the color may be measured, there is still no substitute for a good color-forming reaction, free of unnecessary interferences, side reactions, and extraneous colors.

Step photometers and other similar visual photometers have been widely used abroad and to a lesser extent in the United States. The light intensity is measured by visual observation with this instrument and compared with a calibration curve. Its range of utility corresponds exactly with that of other simple photometers, which include the common colorimeter, in which intensity rather than quality of color is compared, and the photoelectric colorimeter. The instrument is more rapid in operation than the visual colorimeter and is probably somewhat more accurate. A detailed account of its uses is not possible in this review. General treatises dealing with photometry as applied

in microanalyses of biological interest have been published by Krebs (164), Urbach (165), Fretwurst & Maennchen (166), Krumholz (167), and others.

Polarographic analysis.—The polarograph, an instrument developed and described by Heyrovský & his co-workers (168), and extensively discussed by Hohn (169), for automatically measuring and recording current-voltage curves during electrolysis with a dropping mercury cathode, has achieved a degree of development and refinement which amply warrants its careful consideration by biochemists for wide application in microanalysis. Its numerous and varied uses include the analysis of metals, singly or in mixtures; certain anions capable of reduction at the dropping mercury cathode, including nitrate and nitrite (170), bromate and iodate (171), selenite (172); numerous organic compounds, reversibly or irreversibly reducible at the cathode, including carbonyl compounds such as fructose (173, 174), certain simple aldehydes and ketones (175, 176), nitro compounds (177), hydroxylated carboxylic acids (178), reduced sulfur groups as in cystine, cysteine, or proteins (179); and dissolved oxygen (180).

Polarography may be considered as a microtechnique even with fairly large volumes of sample, since the concentration and absolute amount of the constituents determined is usually small. Most determinable components may still be quantitatively estimated at 10^{-4} M, and frequently to considerably lower concentrations. The sample volumes may also be made very small, e.g., down to 0.01 ml., by using properly constructed electrolysis cells. Majer (181) has studied extensively and described such small cells. In the limit, the analyzable quantity is of the order of 10^{-6} millimols or less of the material in question, which compares very favorably with the limiting magnitudes of other highly delicate procedures, and is usually superior to them in accuracy.

The use of the polarograph for automatically plotting current-voltage curves may be substituted by the more laborious point to point observation of galvanometer deflections when known voltages are applied to the electrolysis cell. The use of the dropping mercury cathode for analytical purposes is both rapid and relatively accurate (limiting error about ± 2 per cent). It is, however, attended by the necessity of a reasonable amount of experience and some foreknowledge of the qualitative composition of the sample. A particular mixture of reducible components will usually require a particular adjust-

ment of the sample solution with respect to the pH; the presence of indifferent electrolyte, solvent, etc.; presence of complex forming material; the suppression of maxima in the curves (169), and other factors. In certain cases preliminary separation by chemical means must also be made. The method is particularly advantageous for multiple routine determinations since the development of a valid technique is then justified. Polarography frequently allows accurate analysis of materials not otherwise determinable, or only difficultly determinable (175), especially in the field of organic analysis. The instrument has proved to be quite adaptable to the analysis of trace metals in biological materials.

The broad field of polarography, including a brief treatment of its biological applications, has been excellently reviewed by Kolthoff & Lingane (182), and its application to organic compounds by Müller (183). Complete bibliographies of publications dealing with the method have been compiled through 1938 by Heyrovský (184). Recent applications to the specific field of biological microanalysis include the study of respiration and photosynthesis of *Chlorella pyrenoidosa*, and the respiration rate of yeast, blood cells, and animal tissues through analysis of the oxygen content of the medium (180); the detection of carcinomatous serum (also acute infections) (185); the determination of traces of zinc in small amounts of plant ash (186); the determination of certain ketonic steroid sex hormones in glandular extracts (187), and many other similar types of analyses.

Conductivity.—Micromethods for measuring conductivity are frequently of great utility in the study of urine and sweat excretion, salt balances, and other conditions involving alterations of electrolyte concentrations in small amounts of physiological fluids. For such purposes various types of microcells for conductivity measurements have been designed. Fink & Gross (188) described an easily used and stable cell for volumes of about 0.45 to 0.8 ml. This volume is rather too great for most physiological purposes. Much more suitable is the cell which White (189) used with as small amounts as 0.2 μ l. of solution, an amount readily available for glomerular urine or insect blood studies. This cell has the disadvantage of having removable electrodes which indicates an uncertainty as to its reproducibility. It also requires considerable glass-blowing skill for its construction. The simplest and most convenient type of cell so far described is the pipette type (190) which suffers from few of the defects of the others mentioned, though its present limiting volume is not less than about 5 μ l.

The accuracy claimed is 0.1 per cent with 1 per cent sodium chloride solution. In the reviewer's laboratory, an accuracy of 0.03 per cent has been achieved by use of better measuring equipment. The designers of this cell have used it in the study of excretion of the larvae of *Tenebrio molitor* L. (191).

Osmotic pressure.—The direct micromasurement of osmotic pressures of protein and other physiological solutions, or its indirect evaluation from measurements of vapor pressure, evaporation rate, or other colligative properties, is frequently a useful technique in biochemistry for which highly refined micromethods are available. Of the direct methods, that of Bourdillon (192) appears to have the advantages of small sample volume (0.2 ml.); accuracy (errors not greater than 5 per cent in very dilute solution); ease and rapidity in use. This author applied the technique of direct osmometry to the determination of the molecular weight of human hemoglobin. In general, direct microosmometry may only be used with solutions containing a solute of very high molecular weight, e.g., a protein.

Particularly notable is the rapidly developing use of a thermoelectric procedure originated by Hill (193) which measures the colligative properties by means of differential evaporation rate. This method is not limited to solutes of high molecular weight, but will yield valid data for any type of dissolved material. This rapid and highly precise method required careful attention to details such as temperature control. The original apparatus of Hill, which was extremely difficult to construct, has been largely replaced by instruments such as that of McCracken (194) who described one capable of determining the osmotic pressure of 0.5 μ l. of solution accurately to 0.1 per cent expressed in terms of sodium chloride. Another simplified type of the apparatus was used by Picken (195) in the study of urine formation in invertebrates. This type was applied by Patton & Craig (191) to the study of kidney function and excretory rates of the larvae of the mealworm *Tenebrio molitor* L. The construction and uses of the most recently improved design of instrument have been described by Baldes & Johnson (196). The possible applications of thermoelectric osmometers do not appear to have been widely realized. In addition to the ordinary measurements of osmotic pressure, they are applicable to any study involving changes in the colligative properties of solution which may be caused by enzymic reactions, alterations in water balance, salt metabolism, and a host of other important types of biochemical processes.

Density equilibrium methods.—Density equilibrium methods although virtually unused in the fields of microchemistry or biochemistry until the last three years are rapidly becoming almost indispensable to the solution of certain types of problems. The development of ultramicromethods, due chiefly to Linderstrøm-Lang and his co-workers, has proceeded along two main lines: first, the use of the Cartesian diver as an ultramicromanometer (86); and second, the use of density equilibrium of immersed droplets containing the system being studied, in an immiscible immersion fluid arranged to provide a density gradient, i.e., a dilatometer (85). Both methods depend on the change of the density equilibrium of the floating body immersed in a suitable medium. The first method depends primarily on the change of pressure of a confined gas phase within the floating body, i.e., the diver, and consequently is useful whenever a small biological or chemical system may be made to alter this overlying gas phase. Since this is exactly the field served by the widely used Warburg manometric apparatus, and since the sensitivity of the diver method is about 1500 times as great as that apparatus, many possibilities of application to the microsystem exist. Linderstrøm-Lang & Glick (77) adapted it to the measurement of cholinesterase activity. Boell, Needham & Rogers (80) and Boell & Needham (197) extended its use to the study of the anaerobic glycolysis in the regions of the amphibian gastrula in connection with their extended study of the organization center of the dorsal blastopore. With appropriate modification, the same authors measured the respiratory rates (78) and the respiratory quotients (79) of the same regions of the amphibian embryo. The amounts of tissue used were not usually over 100 μ g. dry weight.

The dilatometric method (85), also perfected on an ultramicroscale by Linderstrøm-Lang and his co-workers, was used by them to measure peptidase activity. In hydrolytic reactions, water was taken up by the reaction and the density underwent a small change, the rate of which was proportional to the rate of the hydrolysis. If the suspended droplet of enzyme-substrate was at equilibrium in a density gradient within a vertical tube, it would rise or fall as its density was lowered or increased. By use of a number of droplets of different known densities in the same gradient, a calibration scale was provided. The relative position of the unknown droplet gave its density, and any change in its position could be measured to determine the rate of change of the density, i.e., the rate of the hydrolysis. While this method may be expected to yield many interesting and important

data, particularly in connection with enzyme reactions in minute systems, little use has been made of it so far.

The dilatometer principle is equally applicable to the routine determination of densities of any solid or liquid material. As a micro-procedure, it has been little used by biochemists or microchemists. Linderstrøm-Lang, Jacobsen & Johansen (198) made use of it in determining the deuterium content of water, which is rapidly assuming importance in biochemistry through the use of deuterium as a chemical label. This material has been habitually determined by micro-density equilibrium procedures (199). Methods similar in principle, e.g., the rate of fall of a drop, have been extensively used, particularly for protein determination in plasma and serum (200).

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INSECT BIOCHEMISTRY

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INTRODUCTION

The biochemist who turns his attention from the traditional study of man and the ordinary experimental animals to the world of insects is likely to encounter many surprising phenomena. This is true for numerous reasons. The vast number of different kinds of insects, their varied habitats and widely contrasting appearances bespeak the millenniums that have elapsed since they diverged from a common ancestor. In many cases the forms during immature and adult life show no resemblance whatsoever. Since the body lies largely within the skeleton rather than around it, this hard outer covering must be cast off at intervals and growth occurs in great spurts at the time of each moult. Since their small size makes it impossible for insects to maintain a constant temperature, they are provided with enzymes, hormones, and other such chemical regulators that function over a wide range of temperature. On the other hand many insects arbitrarily cease growth or reproduction at certain seasons regardless of the particular condition of the environment at the time of beginning or ending dormancy. Adaptation to certain very specialized foods involves the presence of enzymes unfamiliar to mammalian forms. Differing lengths of life cycle, e.g., a few days with certain aphids to seventeen years in the case of the periodical cicada, and reproduction in such varying ways as polyembryony, parthenogenesis, and pedogenesis in addition to the more usual method by no means exhaust the biological wonders exemplified by insects.

It is impossible to touch even in a hasty manner upon all of the lines of investigation, and hence this account is limited to a few fields in which work is most active. Furthermore, it is limited almost entirely to material published between January 1938 and December 1939 but is necessarily not complete for the latter year because of delay in receiving European journals. The reader who desires an account of earlier work will obtain much help from Wigglesworth's *Principles of Insect Physiology* (113) which covers insect biochemistry through 1937 and in some cases into 1938. For convenience, numerous refer-

ences will be made to this text. An account of accomplishments during the period of 1930 to 1935 is given by Hoskins & Craig (62).

The entire subject of the effects of chemicals used for control of noxious insects is in a sense a part of insect biochemistry. It is impossible to summarize recent results in this large subject in the available space. An extended account is in press elsewhere (61).

GROWTH AND METAMORPHOSIS

Growth of larvae.—New or confirmatory information on the nutritional requirements for larval growth has been secured with several insects. In contrast to the results of numerous investigations with flies of similar habits, Di Maria (36) found the larvae of the flesh fly, *Sarcophaga* sp., not to require any vitamin, although it benefits from the B₂ factor of yeast. The chief effect of microorganisms was thought to be their liquefying effect upon the larval food. A comparison of larval growth and size of adults of a species of *Sarcophaga*, also not identified, under nonsterile conditions showed beef thymus to be slightly inferior to beef muscle, and beef thyroid, testicle, and hypophysis to be increasingly unsatisfactory as larval diets (92). The scavenger beetle, *Dermestes vulpinus*, requires one or more components of the B-complex of yeast (50). A previous erroneous conclusion that cockroaches have no need for vitamins has been disproven by McCay (82), who found that *Blattella germanica* needs certain constituents of yeast but is independent of vitamin A or D. The nutritional requirements of larvae of the confused flour beetle, *Tribolium confusum*, are satisfied by a diet consisting of purified casein, corn starch, yeast, cottonseed oil and Osborne-Mendel salt mixture (30). Neither vitamin A nor D, nor the ether-soluble material of yeast are needed. Since all three of the latter insects were raised on solid foods containing but little water, it is unlikely that bacterial or fungal action was of any consequence.

In an exhaustive study of the needs of *T. confusum*, it was found by Frobrich (48) and Offhaus (88) that six substances present in yeast are needed. These include the ordinary vitamins B₁ and B₂, a new substance called B_x which is stable to autoclaving and to alkali and not identical with any known vitamin or bios component, a water-soluble substance of protein nature, and ergosterol and histidine, the latter two occurring together in a slightly soluble combination. As distinguished from the other four substances, histidine and the protein are not essential for maintenance of life, but are needed only for

growth. Vitamin D cannot replace ergosterol. Lack of B₁ or B₂ leads to degenerative changes in the fat body. Excessive intake of B₁ or B₂ is also harmful, resulting in diminution of growth or higher death rate.

As the result of work with *Drosophila melanogaster* larvae on sterile media made from various proteins or from combinations of amino acids, it was concluded that this insect has need of a substance, probably an amino acid, not necessary for mammals (78). Tryptophane and cystine are essential and lysine, arginine and histidine probably necessary. The nutritional needs of the same larva were also studied by Tatum (109) who used a sterile basal diet of acid-hydrolyzed casein (plus a little tryptophane), sucrose and inorganic salts. When this was supplemented with vitamins B₁, B₂, B₆, nicotinic acid, cholesterol, and lecithin it was inadequate, but with centrifuged and filtered yeast autolysate it gave good growth. Eventually three fractions from yeast were secured by making use of their different solubilities in hot ethyl alcohol and different ease of precipitation with barium hydroxide and alcohol. When added singly to the supplemented basal diet given above none allowed more than slight growth but in combination the results were as follows:

Fractions	Fully Grown Larvae	Prepupal Period	Development
I + II.....	6 to 7 days	10 to 11 days	Died as early pupae
I + III.....	12 days	14 to 16 days	Died as larvae or early pupae
II + III.....	9 days	10 to 11 days	Normal adults
I + II + III.....	6 days	6 to 7 days	Normal adults

At present it cannot be decided how many nutritional factors are present in each fraction and the identity of these factors is entirely unknown. In additional experiments omitting various members of the supplementary group it was found that vitamin B₂ (riboflavin) and nicotinic acid are each essential for growth of *Drosophila* larvae.

From somewhat similar experiments with the yellow fever mosquito, *Aedes aegypti*, it was concluded that for larval growth this insect requires vitamin B₁ (thiamin), B₂ (riboflavin), and a third material present in yeast extract, liver extract, or rice polishings extract. This third substance cannot be replaced by nicotinic acid or its amide (111). An ingenious biological test for nicotinic acid has been proposed based on the facts that the larva of the wax moth, *Galleria mellonella*, requires this substance, but only in very small amounts (104).

A final instance of recent work on food requirements for insect

growth is afforded by the honeybee, *Apis mellifica*. When royal jelly is given to female larvae throughout their feeding period they become queens instead of workers. It has been claimed that the tremendous fertility of the queen bee is due to vitamin E in royal jelly, but confirming earlier work, Haydak & Palmer (58) found that there actually is very little of this vitamin in it. Further biological analysis of royal jelly has shown it to be a good source of vitamin B₁ but to have no demonstrable vitamin A or C. Hence the queen bee does not require either vitamin A, C, or E (83).

It will be obvious from the previous discussion that Tatum's (109) statement, that insect larvae need no known vitamin other than those of the B-complex, has not been contradicted by any recent worker. However, factors, as yet unidentified, have been postulated (48, 88, 111). Whether or not these will eventually be identified and proven necessary for mammalian needs also cannot be foretold.

Ecdysis.—The phenomenon of casting off the old outercovering at intervals during the growth of immature insects has long aroused speculations as to the cause and the means by which it occurs. The three leading theories, which apply also to pupation and metamorphosis into the adult form, have been listed as follows (73, pp. 60, 61): (a) All cells of the body have the same rhythm of development. The convincing experiment against this theory is the grafting of hypodermis from a larva about ready to pupate onto a young larva, so that the implanted piece shares all subsequent moults of the host. In this way five extra moults of the wax moth larva, *Galleria mellonella*, have been secured (93). (b) There is a nervous control originating in some part of the central nervous system. The argument against this theory is that many parts of the outer integument appear to be devoid of nerves. (c) There is a hormone elaborated by some tissues and carried throughout the body by the circulating fluid. All modern workers appear to adopt this last viewpoint.

The studies with the tropical bloodsucking bug, *Rhodnius prolixus*, furnish the best proof of hormone control of moulting [cf. (113, pp. 36 to 40) for citations to original papers]. Decapitation during the first few days after a meal prevents the next moult but if the head is left on until after a certain critical time, which varies with each instar, its subsequent removal does not interfere with moulting. Blood from a normal nymph about to moult will enable another to moult even if the latter was decapitated before the critical time and this effect applies to any moult. Hence the same influence is at work for

all moults. Furthermore the effective substance is not specific to the species, for the related bug, *Triatoma* sp., or even the common bed-bug, *Cimex lectularis*, may be caused to moult by injection of blood from *Rhodnius*. Study of various glands during the period of moulting has directed attention to several glands but Wigglesworth (114) has offered proof that the dorsal half of the central brain mass is the essential organ, for when this is taken from a nymph that has passed the critical period and transplanted into a nymph decapitated soon after feeding, the latter is enabled to moult. Severing of the central nerve cord prevents moulting, and from this fact it has been suggested that the hormone is formed by the brain in response to a nervous stimulus originating elsewhere in the body, e.g., from the abdomen as a consequence of pressure resulting from ingestion of a meal. Numerous epidermal cells are concerned in the production of the moulting fluid which at one period spreads throughout the space between the new and the old cuticles and aids in loosening the latter.

In the case of insects which have a complete metamorphosis there appears to be no definite work on the source of the hormone which controls larval moulting. Since certain of these will moult when starved, the initial stimulus cannot be due to distension of the abdomen. Unfortunately, no data are available on the chemical nature or identity of any insect moulting hormone and, in fact, it is idle at present to speculate as to whether there is one or many.

An influence of diet upon moulting is contained in the observation that the larva of the beetle, *Dermestes vulpinus*, grows well but is unable to complete the third moult on a yeast and salt-mixture diet (50). Addition of cholesterol allows normal moulting. Pardi (89) from studies with the beetle, *Melasma populi*, has concluded that the glycogen necessary in forming the new cuticle is elaborated by the eonocytes, which long ago were found to contain this substance, at least at certain times.

Pupation.—In the case of the holometabolous insects the pupal stage is interposed between larval and adult life. Hence two important processes may be distinguished: (a) that by which the last instar larva turns into a pupa; and (b) that by which the adult organs are developed either *de novo* or by modification of the corresponding parts of the larva. The hemimetabolous insects have no pupal stage, and hence only the relatively mild changes associated with the last nymphal moult intervene between the immature and adult life stages.

Earlier workers have shown that removal of the head of several

species of last instar caterpillars prevents pupation if the operation is performed before a certain critical period in the last instar. Similarly, constriction of the body, e.g., of the *Sphinx* moth, before this time results in pupation of only the anterior part, but injection of blood from a prepupa into the surviving larval posterior portion enables it to complete pupation [for references and more extended discussion cf. (101)]. Recent work has been concerned with extension of such experiments to other insects, especially Diptera, attempts to locate the source of the pupation hormone more exactly, and a limited effort to learn something of its properties.

By means of ligatures about *Drosophila* larvae at various times during the last instar, Bodenstein (12, 13) proved that pupation depends upon a center in the anterior portion of the body which exerts its effects about twelve hours before the onset of the pupation process. DeBach (34) has very recently announced similar results with larvae of the housefly, *Musca domestica*, except that the pupation center first exerts its influence about twenty hours before the visible beginning of pupation. With larvae of the wax moth, hypodermis taken from a mature larva and implanted into a young larva undergoes pupation at the same time as its host, and implants of young pupal integument into prepupal larvae undergo a second pupation (94, 95). These results are analogous to the simultaneous moulting of grafted integument or appendages with the host, as mentioned previously. A cytological study of the hypodermal cells of *Ephestia kühniella*, the flour moth, during pupation proved that the first response to the pupation hormone is a great increase in mitosis. This does not occur uniformly even over small areas of the body but in a very irregular manner (77).

For several years various investigators have been removing glands from the head or anterior portion of various caterpillars in an effort to influence pupation, and it was shown that excision of the corpora allata from the head before a certain time in the fourth instar causes the silkworm to pass directly into the pupa, omitting the fifth larval instar (21, 22). It is even possible to cause second instar silkworms to turn into dwarf pupae by the same operation. The higher Diptera do not possess corpora allata but they do have a so-called *Ringdrüse* or Weismann's ring gland. Removal of this gland from *Calliphora vomitoria* larvae two or three days before their normal pupation time prevents pupation, and removal of it from feeding larvae causes them to continue feeding for a prolonged period (26). Sectioning the nerves from the brain to Weismann's ring has the same effect as its

removal. The converse experiment of implanting the ring from mature larvae in young *Drosophila* larvae causes pupation, and the speed and certainty of this effect are greater when three glands are implanted than when a single one is used (55). Implants of brain tissue have no effect. Becker & Plagge (10) have completed the picture by showing that implantation of Weismann's ring into the posterior part of ligated larvae of *Calliphora erythrocephala* enables pupation to occur.

It will be noted from the foregoing discussion that there is some confusion regarding the functions of the hormones which influence pupation, for under somewhat different experimental conditions both prevention and hastening of pupation have been secured. Thus with fly larvae, Weismann's ring has a positive effect whereas with silkworms the corpora allata have an inhibiting influence, but in the case of other caterpillars the effect is again positive. Perhaps the most plausible suggestion is that more than one hormone is concerned in any given insect.

In the case of the bug, *Rhodnius*, it was shown some years ago (113, pp. 45, 46) that the corpora allata furnish a factor which prevents metamorphosis of the nymph into the adult and that removal of these organs allows even first instar nymphs to develop adult characters upon the next moult. The converse of this effect is the moulting of fifth instar nymphs to an additional nymphal instar following the implantation in them of corpora allata from third instar nymphs (115). In contrast to these results is the finding (91) that removal of the corpora allata early in the last nymphal instar from the grasshopper, *Mellanoplus differentialis*, has no effect upon metamorphosis. Koller (73, p. 78) has suggested that in the case of *Rhodnius* there are three possibilities: (a) There is only one hormone and its concentration in the body fluid determines whether moulting or metamorphosis will occur. (b) There are two hormones, one promoting metamorphosis (MeH) and one opposing it (OH). When MeH and OH are both present, moulting occurs but when OH is absent, metamorphosis occurs. (c) There are three hormones, a moulting hormone (MoH) and the two just listed. At moulting MeH and OH neutralize each other leaving MoH, but at metamorphosis MoH and OH neutralize each other leaving MeH effective. Obviously, this is mere guesswork and might be set up in the same manner for the moulting and pupation of holometabolous insects.

An active aqueous preparation of the pupation hormone(s) in

Calliphora erythrocephala larvae has been prepared by a process which includes precipitation of part of the accompanying matter with alcohol (10). The hormone is stable in boiling water and is soluble in water, alcohol, butanol, acetone, acetic acid, and dioxane but insoluble in chloroform or petroleum ether. It is not affected by acid but is destroyed by alkali. Such preparations made from *Lucilia* (Diptera) or *Galleria* (Lepidoptera) larvae are effective in bringing about pupation in *Calliphora* (Diptera). Hence the hormone is not restricted in its action even to an insect order. Exposure of *Lucilia sericata* larvae to 5° C. does not injure the hormone but keeping the larvae at 35 to 37° C. for two hours causes delay in pupation, apparently due to destruction of part of the hormone (85).

An analysis of the forms of phosphorus during the course of pupation of *Calliphora erythrocephala* showed that the pupa has less lipid phosphorus, less alcohol-soluble phosphorus and more inorganic phosphorus than the larva (71). Also the phosphorus-containing nucleic acids of this insect differ in certain properties of solubility and stability from the ordinary nucleic acids. An increase in nucleic acid in bands close to the heterochromatic regions of the salivary gland chromosomes of *Drosophila* has been reported (28) and a relation to gene reproduction has been suggested.

The effect of food intake upon pupation of *Drosophila melanogaster* has been studied (7). If taken from a standard food of cornmeal, molasses, fresh yeast, and agar at any time up to seventy hours after egg hatching, the larvae fail to pupate, but if removed shortly after seventy hours, pupation and eclosion occur. Of course the size is less the shorter the feeding period, e.g., with the minimum time of seventy hours the resulting flies were only one eighth as heavy as normal adults. Temporary removal from food at any time up to the critical period results in delay of pupation somewhat more than proportional to the starvation period. This indicates that during complete starvation the processes that lead to pupation not only stop but are reversed to some extent, i.e., metabolic activities during the fasting period destroy some of the hormone or some of its precursors.

Differentiation of imaginal organs.—During the pupal period the various organs of the adult insect body assume the position, form, size, color, and other properties which with certain minor alterations, characterize them during the rest of the insect's life. In the light of the role which hormones play in the processes of moulting and pupation it is to be expected that similar influences will be at work during

imaginal differentiation. It is easily observable that in the pupae of holometabolous insects the visible differentiation of the adult body begins in the thorax region and spreads from there both forward and backward. Earlier workers had confirmed the presence of a differentiation center in the thoracic region of the pupa by ligaturing, exposure to x-rays, grafting, centrifuging, etc. [for review see (101)].

Bodenstein (12, 13), working with *Drosophila melanogaster* and with the California oak moth, *Phryganidia californica*, found by ligation technique that development of the adult abdominal ectoderm depends upon a substance from the anterior part of the pupa which is present for only a limited period during pupation. The impulse is apparently dependent upon continuity of the ectoderm, for an abdomen joined to a thorax through a glass tube does not develop, whereas if grafted it does. A new viewpoint on these phenomena was given by the later discovery (14, 15) that differentiation into imaginal ectoderm on abdomens ligatured before the critical period is greatly promoted by exposing them to pure oxygen instead of ordinary air. Similarly, differentiation of eye disks implanted into ligatured abdomens is favored by oxygen. Since it has been shown (47) that oxygen diffuses through the skin of blowfly larvae, Bodenstein assumed that oxygen will also penetrate into pupae and act as the, or at least one of the, essential factors for differentiation. He was able to correlate the known facts regarding the critical period with the development of the thoracic tracheal system and suggested that this structure may be the differentiation center. Bodenstein regards his work as reopening the question of the existence of hormones for the particular differentiations which he studied. It may also be suggested that the hormone may be formed in an inactive condition and require oxidation before it is effective.

In certain mutants of *Drosophila* and of *Ephestia kühniella* the development of eye colors has been found to be correlated with the presence or absence of certain genes (4, 42). The genes act through particular organs which elaborate the hormones required for development of the given colors. Thus a substance called v^+ is formed by the eyes, the Malpighian tubes, and the fat body of all mutants of *Drosophila* except the vermilion-eyed. Another substance, cn^+ , is present in the eyes and Malpighian tubes of all except the mutant cinnabar. A substance called A-hormone has control of color of eye, brain, and testes sheath in *Ephestia* and is present in all these organs of the wild race.

Studies on the chemical properties of the v^+ and cn^+ substances obtained from dried wild type *Drosophila* pupae have been reported (10, 70, 110). The two hormones are so much alike that no differences were detected. They are soluble in water and alcohol but insoluble in chloroform, ether, benzene, or acetone. In butyl alcohol the solubility is at a maximum at about pH 6.0, from which it is judged that the compounds are amphoteric. They are precipitated by ammoniacal lead acetate, by alcoholic barium hydroxide, by phosphotungstic acid, and by mercuric acetate plus sodium carbonate, which is supposed to be specific for amino acids and their derivatives. The molecular weight (calculated from diffusion through agar blocks) is approximately 500. The hormones are rapidly destroyed by alkali but they are stable to 1 *N* sulfuric acid for at least one-half hour. Intracellular enzymes from *Drosophila* pupae inactivate the hormones in the presence of air, as does iron oxide. The hormones are stable as dry or liquid extracts or in dried pupae at ordinary temperatures and for one hour at 100° C., but are all destroyed in one hour when the pupae are heated to 160° C. It may be tentatively concluded that these substances are derivatives of one or more amino acids whose identity must await isolation of the active compounds.

The hormones v^+ and cn^+ are absorbed and produce their characteristic effects in *Drosophila* when given with the food of test larvae. Crushed pupae are a convenient source of the hormones but they must be heated for a short time to inactivate the destructive enzymes. By this technique both v^+ and cn^+ have been found to be present in a large number of insects, for among representatives of nearly thirty families in thirteen orders certain representatives of only six families failed to respond to the test (6, 80). Of course, other members of these families may react positively. The two substances occur in widely varying ratios among the various families. The ready conversion of v^+ hormone to cn^+ is shown by the experiment in which larvae of the vermilion-eyed mutant were fed upon a source of v^+ hormone and after these had pupated they were fed in turn to larvae of the cinnabar variety (108). Flies from these showed a distinct eye-color change.

As was mentioned earlier the A-hormone of *Ephestia* plays much the same role as the two eye-color hormones of *Drosophila*. The distribution of A and v^+ substances in various insects leads to the suggestion that they are chemically identical and that the genes responsible for them are homologous (5, 96). No systematic search for

these color-producing hormones seems to have been made with animals, but it has been found that they do not occur in sheep brain (69), in embryonic mouse tissue (80), or in yeast (110), but are present in the crab *Uca* (42).

During the study of the effect of larval feeding upon pupation it was noticed that partial starvation of the vermilion-eyed mutant of *Drosophila* in some instances led to a darkening of the eyes, i.e., the effect of feeding v^+ hormone was obtained (7). Complete starvation either for short or long periods had no effect, but if partial feeding (e.g., from a very dilute yeast-agar mixture), prevails during the sixty- to seventy-hour period of normal development at 25° C. at least a hundredfold increase in v^+ hormone may be produced in the mutant vermilion. Similar results were obtained by Khouvine *et al.* (70), who added the information that addition of small amounts of yeast to the normal diet or the feeding of peptones which contain tryptophane also cause darkening of the eyes of either vermilion or cinnabar flies, but that addition of glucose suppresses the effect. All these observations together with the amino-acid-like nature of the eye-color hormones suggest that in the normal metabolism of these flies the precursors of the v^+ and cn^+ hormones are used for protein synthesis instead of hormone synthesis. But under conditions of low food intake some breakdown of protein may occur with consequent opportunity for hormone synthesis. This suggestion still leaves unexplained the lack of effect from total deprivation of food at intervals.

In repeating some of the work just described (108) it was found that addition of tryptophane to the diet of larvae of the vermilion-eyed *Drosophila* did not cause darkening of the eyes unless a certain unidentified *Bacillus* sp. was also present. A preparation from the bacteria-tryptophane culture was also effective by injection, but again if the *Bacillus* was grown without tryptophane, it could not elaborate the substance that had the effect of v^+ hormone. Since the active bacterial preparation and all active preparations from *Drosophila* pupae have a yellow color, it is likely that the color is associated with the effective substance and that v^+ hormone is formed by the micro-organism.

Mention should be made of biochemical investigations on one other organ during imaginal differentiation. The development of pattern on insect wings has long been a matter of much interest and a comprehensive theory based upon the idea of developmental processes occurring at different rates in different parts has been restated in detail

by Goldschmidt (52). Experimental proof in the case of the three Lepidopterons, *Ephesia kühniella*, *Platysamia cecropia*, and *Papilio ajax*, has been offered by Braun (24). Wings removed from developing pupae at a certain time characteristic for each species, before pigmentation can be seen, assume when dry a relief pattern corresponding in every detail with the adult wing pattern. The relief effect results from the shrinking of those scale cells which at the time are still soft and filled with liquid, whereas those which are already chitinized do not collapse. These latter scales cannot take up pigment when it appears later and, consequently, they remain light in color, whereas the soft cells are able to become pigmented. This theory was checked and the adult wing patterns were obtained by soaking pupal wings in a saturated solution of tyrosine which after entering the permeable scale cells was oxidized to a melanin pigment by aid of the tyrosinase which seems to be present always in insect blood and cell fluid. Additional confirmation of the differences in chitinization was gained by treating the unpigmented pupal wings with iodine and zinc chloride solutions. Heavily chitinized regions are stained a violet-brown color, more lightly chitinized regions a light brown, and again the adult patterns were reproduced.

Diapause.—Many insects are characterized by a cessation of growth or development at some stage of the life cycle, this arrest being more or less independent of environmental factors at either its beginning or conclusion. This behavior affords a means for surviving unfavorable conditions, especially cold or dryness, and hence is of enormous practical significance in the case of harmful species. Accordingly, it has received a great deal of attention for many years [for summaries of previous work see (101, pp. 186-191), and (113, pp. 67-70)].

The work on diapause of the embryo of the grasshopper, *Melanoplus differentialis*, has been continued by Bodine and his co-workers. In the winter generation of this insect, development of the embryo ceases before blastokinesis occurs and no further cell division occurs until spring. While efforts to relate respiration, peroxidase, and tyrosinase content of the egg or embryo to diapause have yielded no definite results, new information has been gained recently on the properties of the tyrosinase. It is obtained in an inactive form, i.e., as protyrosinase, by grinding, washing, and centrifuging the egg. Dialysis at 0° C. with a cellophane membrane gave the unusual result that as the activity (as measured by consumption of oxygen in presence

of tyramine hydrochloride) increases, the solubility declines (100). Protyrosinase in the egg is activated by a substance as yet unidentified which is soluble in oil and in glycerol. It is also activated by sodium oleate, urethane, urea, acetone, and by exposure to temperatures up to 65° C. Above this temperature it is destroyed rapidly (16). During the embryonic development of *M. differentialis*, protyrosinase is formed at a rate corresponding to a simple autocatalytic reaction with a fixed amount of substrate. The maximum rate of increase occurs at about the sixteenth day of development at 25° C. (1). The natural activator is present at maximum amount during early development of the embryo, falls very rapidly until diapause sets in, remains constant until a few days after diapause is over, and then declines to nearly zero by the time of hatching (18). There is no obvious correlation between the activity of the activator and the occurrence of diapause.

Catalase activity is reduced in diapause silkworm eggs (87) and in overwintering larvae of the pine beetle, *Dendrolinus pini*, according to Emchuk (41). In a study of the relation of enzymes to cold-hardiness, Kozhanchikov (75) found the dehydrase enzymes, which enable anaerobic respiration to proceed, to be correlated with the occurrence of unsaturated fatty acids apparently peculiar to insects. The quantity of total fat shows no relation to thermostable respiration nor to cold-hardiness.

METABOLISM

While the food requirements of insects are met by the usual carbohydrates, fats, and proteins, these substances may be in a form indigestible by a vertebrate animal, e.g., as cellulose or keratin. One finds enzymes peculiar to the insect either elaborated by it or by symbionts. Digestive enzymes are produced in part by the salivary glands but mainly by the cells lining the intestine and its ceca. All the cells may act in unison, in which case they are said to be synchronous, and a given cell may have a single (monophasic) or several (polyphasic) periods of activity. In carnivorous insects, the enzyme production is usually monophasic and synchronous, while in herbivorous, it is polyphasic and asynchronous (97). Absorption of the split products is accomplished by the same cells and follows enzyme production in *Dytiscus marginalis* (39). In addition to digestive enzymes, the salivary glands or gastric ceca in some bloodsucking insects as mosquitoes or *Rhodnius* may secrete a hemagglutinin and anticoagulin (35).

The present trend of enzymatic studies is toward a correlation be-

tween the chemical constitution of the material and the specificity of enzymes found. Thus Hopf (60) showed that the ash-bark beetle, *Hylesinus fraxini*, may get its proteins directly from the bark and its carbohydrates from the simple sugars, disaccharides, α -glucans, and the hexosan parts of the hemicelluloses present in the bark. The enzymes present are a tryptase, peptidase, saccharase, lactase, maltase, amylase, and galactanase. As there is no hemicellulase present in this beetle, it is postulated that the enzymes present split off the hexose units and this process accompanied by oxidation accounts for the change between ingested food and excreta. The honeybee produces an enzyme thought to be an α -glucosidase capable of hydrolysing melizitose (54). The normal diet may not always be an optimum. Thus the beetle, *Hylotrupes bajulus*, although able to digest proteins from wood, grows much faster when peptone is added to its food (9). The addition of sucrose to the mulberry-leaf diet of silkworms has been shown by Kato (67) to influence the activities of invertase, lipase, phenolase, and catalase, but amylase activity (65) is unchanged. The flour beetle, *Tribolium confusum*, is unable to digest sucrose probably because of a lack of invertase (30). *Tenebrio molitor* larvae grow well on starch, mannite, and levulose; less well on glucose, saccharose, xylose, laclose, glycogen, and arabinose; and fail to grow on galactose or inulin (79).

The role played by symbionts in digestion as well as in synthesis of accessory foodstuffs has received some attention. Simmons (107) has shown that in *Hypoderma lineatum* (Diptera) glycogenase, lipase, trypsin, and erepsin are secreted by the larva while lactase, maltase, invertase, and rennin are elaborated by the bacterial symbionts. However, the blowfly larva, *Lucilia sericata*, which often infests animal wounds and was used a few years ago in treatment of osteomyelitis, excretes urease in the absence of contaminating microorganisms (103). The ammonia formed by breakdown of urea accounts for the alkalinity of infested wounds. An interesting symbiotic relationship exists in *Zootermopsis* (Isoptera). Hungate (63) has shown that the protozoa in the hind gut of this insect digest the wood ingested by the termite and metabolize it anaerobically forming acetic acid, carbon dioxide, and hydrogen. The termite absorbs the acetic acid and oxidizes it. The rest of the carbohydrate and protein requirements are met by digesting both fresh wood and reingested excreta.

While insects vary greatly as to their efficiency as converters of foodstuffs to body substance, some of them are much more efficient than mammals. In the immature cockroach, *Blattella germanica*, Mc-

Cay (82) studied the utilization of a dry skimmed milk and ground whole-wheat diet. Evans (44, 45, 46) has studied the utilization of the normal food of the larval Lepidopterons, *Phalera bucephala*, *Malacosoma neustria*, *Aglais urticae*, *Pieris brassicae* and the beetle, *Tenebrio molitor*. The results have been summarized in Table I for

TABLE I

UTILIZATION OF FOODSTUFFS EXPRESSED AS PERCENTAGE OF SUBSTANCE
IN DIET BASED ON DRY WEIGHT

	<i>Phalera bucephala</i>	<i>Malacosoma neustria</i>	<i>Aglais urticae</i>	<i>Pieris brassicae</i>	<i>Tenebrio molitor</i>	<i>Blattella germanica</i>
Water	60	32	22	19
Dry matter	35	34	26	33	46	31
Protein nitrogen	56	72	..	84	54	..
Rest nitrogen ..	48
Fructose	70	46	..
Glucose	80	64	50	58	67	..
Sucrose	93	88	73	63	96	..
Starch	35	9	0	59	..
Fat	63	27	55	..	83 (approx.)	..
Calcium	10
Phosphorus	25
Nitrogen (total)	29	..	55	..	60

which some values have been calculated to percentage from the authors' data. The values are based on the amount of substance in the ingested food and in the excreta and, therefore, represent the percentage of the substance removed from the food without regard as to how it was metabolized.

The intermediary metabolism of insects is little known but some points of interest have been recently reported. In the corn-ear worm, *Heliothis obsoleta*, the glycogen content falls from 7 per cent to 3.5 per cent (dry wt.) during the transition from larva to pupa and remains sensibly constant throughout pupal life; the rate of decrease is proportional to the temperature (37). Fat can be synthesized by the cockroach (82) and the iodine number is lower than that of the ingested fat. In the corn-ear worm, the iodine number of stored fat is lower than diet fat and decreases from 79 to 66 during the pupal period at a temperature of 86° F., indicating that mostly unsaturated fats are used. At a lower temperature (40° F.) there was little change in iodine value. The saponification number of the fat is about 200. The total fat content is from 45 to 50 per cent of dry weight, and consists of one third saturated fatty acids and two thirds unsaturated acids, having an iodine number of 100 (38). When sucrose is added

to the mulberry-leaf diet of the silkworm (66) 8.5 per cent is converted to glycogen, 40 per cent to fat, and 52 per cent is oxidized.

Knowledge of the degradation of protein is obtained largely from the excretory products. Except in a few instances, the gut of the insect is not sterile so that there is some uncertainty as to the role of the intestinal flora and fauna. Evans (44) found the distribution of nitrogen in excreta of *Phalera bucephala* to be uric acid nitrogen 0.16 per cent, ammonia nitrogen 0.17 per cent, amino nitrogen 0.03 per cent, and total nitrogen 2.34 per cent of the dry excreta. The nitrogenous metabolism of the flesh fly, *Lucilia sericata*, has been studied by Brown (25). The larvae were grown on a sterile casein, yeast, salt mixture, and lanolin diet, and insects and excreta analyzed throughout the life cycle. The nitrogenous materials excreted by this insect are uric acid, allantoin, ammonia, urea, creatine, and creatinine. The results are summarized in Table II. It is unfortunate that no dry weight data were given since it is not possible to compare the amounts from age to age. Bottero (20) showed that injection of urea into blood of silkworms made little change in their metabolism but the ammonium salts—ammonium hydroxide, sulfate, formate and carbonate, especially the last—caused serious disturbances. The ammonium salts are partly excreted and partly converted to urea.

The mechanism of excretion has been studied in *Tenebrio molitor* larvae (90). In this insect the Malpighian tubules act as a semipermeable membrane passing an ultrafiltrate of the body fluid into the tubules. This filtrate passes down the hind intestine to the rectal region where a certain portion of the solutes is returned to the blood. The rate of filtration by the tubules is 2.2 c.mm. per hour.

RESPIRATION

One of the peculiarities of insects is their respiratory system which consists of a network of tracheae opening to the outside at two or more spiracles and inwardly becoming smaller until at a diameter of a few microns they change their structure and become minute tracheoles. The tracheoles are frequently filled with a fluid and are so numerous that nearly every cell is contiguous to one. If the oxygen need is very great a cell may be penetrated by a tracheole. Previous to hatching the tracheal system is filled with fluid which is absorbed at the time of eclosion when the spiracles are exposed to the air (112), which suggests a possible nervous mechanism. This initial removal of fluid from the tracheal system at eclosion, and in the mosquito

TABLE II
NITROGEN CATABOLISM IN THE FLESH FLY, *Lucilia sericata*

	IN EXCRETA					
	4-Day Larvae	Prepupa	Meconium	1-Day Adult	3-Day Adult	6-Day Adult
Uric acid*	0	0	24 mg.	1 mg.	3 mg.	8 mg.
Allantoin*	0.5 mg.	1.6 mg.	3 mg.	0.5 mg.	1.2 mg.	2 mg.
Ammonia*	22 mg.	0	0.25 mg.	0.5 mg.
Creatine	0.10 mg./gm.	0.15 mg./gm.
Creatinine	0.05 mg./gm.	0.09 mg./gm.
Urea	0.03 mg./gm.	0.07 mg./gm.
	IN INSECTS					
	Egg	4-Day Larvae	Prepupa	5-Day Pupa	1-Day Adult	3-Day Adult
Uric acid*	trace	0.5 mg.	27 mg.	2.5 mg.	1.0 mg.
Allantoin*	trace	0.7 mg.	3 mg.	0.5 mg.	1.0 mg.
Ammonia*	0	0	0	1.4 mg.	0.05 mg.	0.10 mg.
Creatine	0.09 mg./gm.	0.08 mg./gm.	0.9 mg./gm.	0.05 mg./gm.	0.23 mg./gm.
Creatinine	trace	0.03 mg./gm.	0.5 mg./gm.	0.02 mg./gm.	0
Urea	0.1 mg./gm.	0.02 mg./gm.	0.1 mg./gm.	0.03 mg./gm.	0.11 mg./gm.
Uricase	+	+	—	—	+	+
Urease	—	—	—	—	—	—
Allantoinase	—	—	—	—	—	—

* All data on these substances per 100 individuals of unknown weight. Other substances given in mg. per gm. wet weight of excreta or insect respectively.

larvae at each moult, is probably an active absorptive process of the cells composing the tracheal system.

It has long been known that the integument of insects is somewhat permeable to oxygen and carbon dioxide. Recently, proof has been given that if the oxygen tension in the tissues of blowfly larvae is made very low, by ligating the anterior and posterior spiracles, nearly one fourth of the normal basal oxygen consumption occurs through the skin (47). It is calculated that perhaps one tenth of the normal basal oxygen consumption occurs through the skin. In the normal terrestrial insect, the amount of oxygen admitted to the tracheal system is controlled by respiratory movements and by the opening and closing of the spiracles. In the flea there is a rhythmic pulsation of the tracheae accompanied by a rhythmic opening and closing of certain of the spiracles (59). High oxygen concentrations in the inspired air slow the rhythm and increase its amplitude. Carbon dioxide above 6.5 per cent stops the rhythm immediately. The higher the concentration of carbon dioxide, the longer the time before resumption of pulsation after replacement of the gas with normal air.

There is still no evidence of an oxygen carrier generally present in insects, although it has been shown that insect blood removed from the body can absorb oxygen for a short time at a high rate to an extent of 150 volumes per cent (76).

One of the favorite methods of studying metabolism is by determining the respiratory quotient. In an insect there may be some question as to the significance of the R.Q. unless the metabolism is in a steady state which usually is difficult to obtain. The *Agrotis segetum* (Lepidoptera) female shows a rise in R.Q. from 0.76 (starvation for twenty-four hours) to 1.7 two hours after a glucose meal (74). The R.Q. of the queen honeybee is lower (0.87) during the pupal stage than that of the worker which is 0.96. During the larval stages the R.Q. for both may rise above 1.0 (84). In both cases cited an R.Q. above 1.0 is interpreted as measuring the conversion of carbohydrate to fat. The R.Q. of grasshopper embryos may be raised from a normal of 0.75 to 0.91 by dinitrophenol which causes increased oxidation of protein with increased production of ammonia (17).

The amount of oxygen required and the partial pressure of oxygen required for normal life vary greatly. In prepupae of *Drosophila melanogaster* the amount of oxygen consumed varies from 2.5 c.mm. per mg. per hour for individuals weighing 0.9 mg. to 2.1 c.mm. per mg. per hour for those weighing 1.3 mg. (40), with no difference resulting from sex or genotype. The minimum amount of oxygen in

the air requisite for mobility of six *Drosophila* species varied from 1.60 per cent for *D. melanogaster* to 2.80 per cent for *D. obscura*. These values were found at the optimum breeding temperatures (31). In *Calliphora erythrocephala* (Diptera) larvae the oxygen consumption increases from a basal value, secured by ligaturing, of 0.47 c.mm. per mg. per hour to a normal active value of 0.90 c.mm. per mg. per hour at 27° C. (47). In the Japanese beetle starvation causes a drop in oxygen consumption from 6.5 c.mm. per gram per minute (0.39 c.mm. per mg. per hr.) to 1.92 c.mm. per gram per minute (1.15 c.mm. per mg. per hr.) at the end of twenty days. Most of the decrease occurs in the first three days. The R.Q. falls from 0.78 to 0.70 (11). Oxygen consumption determinations have been used to study the effect of dehydration on grasshopper eggs (105). Considerable oxygen may be used in pigment formation during certain stages of early adult life in the mealworm (106).

Keilin (68) has demonstrated the presence of cytochrome- a_3 in the flight muscles of bees and other insects. This oxidase combines with carbon monoxide, potassium cyanide, hydrogen sulfide, sodium azide, hydroxyl amine and probably sodium fluoride and ethyl alcohol. A further study of the cytochrome complex may explain the action of inhibitors on the cellular respiration of insects. *Drosophila* pupae have a lower oxygen consumption in the presence of carbon monoxide. The residual respiration, n , obeys Warburg's equation, $[n/(1 - n)] [CO/O_2] = K$, showing that the oxidizing enzyme system is saturated. Strong light decreases the effect of carbon monoxide (116). Ultraviolet light is known to stimulate growth which is reflected in increased oxygen consumption of grasshopper eggs (99). An inhibition of as much as 90 per cent of the respiration of grasshopper embryos by cyanide is not necessarily fatal (102). Pyocyanine increases the respiratory exchange in the grasshopper embryo by about the same amount in normal and in cyanide- and carbon-monoxide-inhibited individuals. Carbon monoxide inhibition is not additive to carbamate or veronal inhibition. A correlation of these results with those of Keilin requires further work (27). Different parts of the Bruchid (Coleoptera) egg show a varying susceptibility to potassium cyanide during development as does the grasshopper egg. In the Bruchid egg the effect of potassium cyanide on the several germ layers has been studied and the implications concerning the progressive embryonic organization noted (23). The effects of oxygen deprivation on the formation of imaginal structures during the pupal stadium has also been described (49).

Cold-hardy forms, such as prepupae of *Coresus septentrionalis* and eggs of *Lymantria dispar*, are characterized by thermostable respiration and by possessing a chloroform-insensitive component of their respiration, whereas in nonhardy insects respiration is thermolabile and chloroform-sensitive. These facts together with the action of various substances on diapause grasshopper embryos may be explained by assuming that cold-hardy insects use dehydrases and nonhardy use oxidases in their cellular respiration (75). The temperature coefficient, Q_{10} , for isolated grasshopper embryos in the prediapause and diapause states is higher than the Q_{10} of the intact eggs. At low temperatures (15° to 25° C.) the Q_{10} for diapause eggs is less than that for prediapause or postdiapause eggs. At high temperatures (25° to 35° C.) the Q_{10} is the same for prediapause and diapause eggs. Postdiapause eggs are temperature-insensitive (53).

The susceptibility of insects to carbon dioxide intoxication is probably very variable. The carbon dioxide in termite colonies, *Eutermes exitiosus*, is nearly fifty times that in normal air, about 1.5 per cent (33), while the flea, *Xenopsylla* sp., tolerates 6.5 per cent (59). A concentration of 100 per cent carbon dioxide causes death of the beetle, *Phytododues plicatus*, after four days (98).

Chironomus larvae may live anaerobically for a time by converting glycogen to fat but must have an "oxybiotic" recuperation. The oxygen produced by the glycogen-to-fat synthesis may be used during recuperation in secondary oxybiosis (57). The secondary oxybiosis is the portion of the respiration dependent upon partial pressure of oxygen (56). *Ephemera vulgata* (Ephemeridae) larvae show a secondary oxybiosis but *Cleon dipterum* larvae do not.

BLOOD

Despite the fact that three reviews on insect blood have recently appeared (51, 81, 86) this phase of insect biochemistry lags behind other subjects. The oxygen capacity of blood of *Prodenia eridania* (Lepidoptera) is about two volumes per cent (2, 76). The blood of this insect contains a lipase and an invertase but no other digestive enzymes. The twenty-nine constituents accounted for occur approximately in the proportions found in other insects. The blood of the silkworm contains urea throughout larval life (19).

The osmotic pressure of the blood of *Tenebrio molitor* larvae is equal to that of a solution containing about 2.12 per cent of sodium chloride and that of *Pieris rapae* to a solution containing 1.35 per

cent of sodium chloride (90). Terrestrial insects presumably maintain their osmotic pressure by water resorption from the gut, but aquatic forms may use a different method. Both *Culex* and *Chironomus* larvae are able to absorb sodium chloride parenterally through the anal papillae from the concentrations ordinarily found in fresh water (72). Further studies on larval blood of *Culex pipiens* and *Aedes aegypti* show that the chloride ion content of *Aedes* is the same as a 0.30 per cent solution of sodium chloride and for *Culex* a 0.28 per cent solution. The osmotic pressure for both corresponds to a 0.80 to 0.89 per cent solution of sodium chloride. The concentration in the blood remains constant until the sodium chloride content of the external medium reaches 0.65 to 0.75 per cent; above this concentration chloride enters, and in solutions containing 1.6 per cent of sodium chloride the larvae die as a result of hypertonicity (112). The regulatory ability of the salt-water mosquito, *Aedes detritus*, must be even greater since the larvae can tolerate distilled water, 7 per cent sodium chloride, and 0.05 N sodium hydroxide. They can not survive in solutions of chlorides of potassium, calcium, or magnesium of an osmotic pressure equivalent to 3.5 per cent sodium chloride. The salt balance is maintained by the gut epithelium since the gills and integument are not involved (8). Salinity tolerances have been studied for other mosquitoes (64, 117). An interesting application of this work is the use of salt solutions for demonstrating physiological differences between the larvae of certain European Anopheline mosquitoes which are very similar in appearance (3).

The complement fixation reaction using rabbit sera immunized to saline extracts of several species of *Drosophila* can be used to differentiate the antigens of the species although inconclusive results were obtained in some instances (32). The possible applications of this to systematic entomology and phylogeny are evident. Although the functions of the cellular constituents of insect blood are as yet but little understood, there has been considerable interest in the cytology of these structures (29, 43).

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APPLICATION OF RADIOACTIVE INDICATORS IN BIOLOGY¹

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Natural radioactive isotopes of some of the heaviest elements are easily available and labelled lead, bismuth, and thorium were used in biological research at an early date. The circulation of lead in plants (1) and that of lead, bismuth, and thorium in both normal (2, 3) and carcinomatous (4) animals was investigated by this method. Extended use was made of radioactive bismuth in the study of the rate of absorption of the various bismuth preparations used in syphilis therapy (5). The discovery of artificial radioactivity and the rapid and successful development following this important event (6) made it possible to obtain "artificial" radioactive isotopes of numerous elements and opened thus a vast field for the application of isotopic indicators in biology. Among the elements of biological importance, hydrogen has no radioactive isotope, while nitrogen, oxygen, and carbon have such short half lives that their application for most purposes is excluded, though the radioactive isotope of carbon has met with some very useful applications (see pp. 658, 659). The discovery of heavy hydrogen and the recent progress (7) in the separation of the isotopes of oxygen, nitrogen, and carbon have made it possible to make use of labelled hydrogen, nitrogen, oxygen, and carbon, the tagged elements being identified by density measurements and mass-spectrographic determinations respectively. No fundamental difference is to be found between the application of radioactive and of stable isotopes as indicators. To limit the scope of discussion, however, it may be preferable to treat the application of radioactive indicators separately from that of the nonradioactive ones. In what follows, we will first discuss the use of radioactive phosphorus for labelling purposes. This isotope has found so far the most extensive application; furthermore, in view of its importance both in the study of inorganic and of organic metabolism, its application presents very suitable examples of the type of problems which can be successfully attacked by the use of tagged elements.

¹ In this review papers published prior to November 1, 1939, are considered.

PHOSPHORUS

Preparation and general considerations.—The active phosphorus used as an indicator, P^{32} , has a half life of 14.5 days and emits β -rays, the intensity of which is cut down to half of its initial value by an aluminum foil about 0.5 mm. thick. In all the early and some of the later investigations P^{32} was prepared by the action of fast neutrons emitted by mixtures of radium and beryllium on large amounts of carbon disulphide (8). Under the action of γ -rays emitted by radium a slight decomposition of the carbon disulphide takes place; the decomposition products, which adsorb most of the P^{32} , partly settle on the wall of the vessel and partly remain suspended in the liquid. The active phosphorus is removed by treating carbon disulphide, which has been irradiated for a week or two, with quite dilute nitric acid. The walls of the glass container, after removal of the carbon disulphide, are treated in the same way. An alternative method is the removal of the P^{32} in an electric field (9) on copper electrodes immersed in the carbon disulphide during the neutron bombardment. The weight of P^{32} obtained is very small (about 10^{-10} gm.). It is converted into sodium phosphate and is administered as such, or after "dilution" with nonactive phosphate. Much stronger preparations than those mentioned above, which have an activity of several millicuries, are obtained by the action on red phosphorus of accelerated deuterium ions produced by a cyclotron (10). Most of the later work with labelled phosphorus has been carried out by such cyclotron phosphorus, supplied by the Radiation Laboratory at Berkeley, not only to workers in that University, but also to those working in different institutions in the United States and in Europe. The Geiger counter (11, 12) or, if strong preparations are available, a sensitive electrometer (13), is used to determine the activity of the sample being investigated. To avoid corrections for the decay of the P^{32} content of the sample with time, the activity is often determined relative to that of a standard preparation of P^{32} decaying with the same period. Usually an aliquot of the active sodium phosphate solution to be administered is kept as a standard. To avoid corrections for the difference in the absorption of the β -rays emitted by samples of different thickness and composition, the following procedure is often employed: The sample to be investigated is incinerated; one aliquot of the solution obtained is used for the colorimetric determination of the phosphorus content of the sample, while to another aliquot a comparatively large, known amount of inactive sodium phosphate is added and the mixture of the active and inactive phosphate precipitated as ammonium magnesium phosphate. The ac-

tivity of the precipitate obtained is compared with that of the standard solution treated in the same way. This procedure permits comparison of the activity of samples having the same composition and the same weight (20). In numerous investigations which deal with the application of P^{32} the percentage of the labelled phosphorus administered, which is found after the lapse of different intervals in the various organs, and compounds isolated from the latter, is determined. By such investigations what percentage of the phosphorus atoms administered is incorporated after the lapse of a day, for example, in a tooth or in the creatine phosphoric acid isolated from the muscles can be determined. Often, however, the main problem is not the one mentioned but the determination of what percentage of the creatine phosphorus, lecithin phosphorus, and phosphorus of other compounds isolated from the organs became labelled a certain time after the administration of the active phosphate.

The phosphorus of organic phosphorus compounds does not exchange with other phosphorus atoms present in the same phase. When shaking a solution of hexosemonophosphate (14), glycerophosphate (15), lecithin (48), casein (18), or nucleic acid (21) in the presence of labelled phosphate no labelled organic phosphorus compound is formed. The formation of the latter necessitates the synthesis of the compound in question from components including labelled phosphate. The presence of labelled lecithin, for example, in one of the organs of an animal proves that the labelled molecules of this compound were synthesized in the body after the administration of the labelled phosphate. By determining what percentage of the lecithin, isolated from an organ, became labelled after a certain time, the rate of renewal of lecithin in the organ in question is arrived at (20). Such determinations necessitate the determination of not only the P^{32} content of the sample, but its P^{31} content as well. Data are thus obtained on the activity per mg. of phosphorus which is denoted as specific activity. How the specific activity compares with the activity per gm. of fresh tissue is seen in Fig. 1 (21). A glance at the figure shows that only a minute part of the skeleton or brain phosphorus, but a comparatively large part of the liver and kidney phosphorus, is renewed within four hours.

Blood phosphorus.—Most of the P^{32} atoms introduced into the plasma soon leave it. In the human plasma out of one hundred labelled phosphate ions only two are present after the lapse of two hours (20), the other phosphate ions having been replaced by fresh ones located formerly in the diverse organs. The loss of labelled phosphate is

partly due to its rapid diffusion into the extracellular space of the body. As the volume of the latter is about seven times larger than that of the plasma, this dilution will bring down the P^{32} concentration of the plasma to one-eighth of its original value. The further loss is due to exchange with phosphorus atoms present in the bones and in

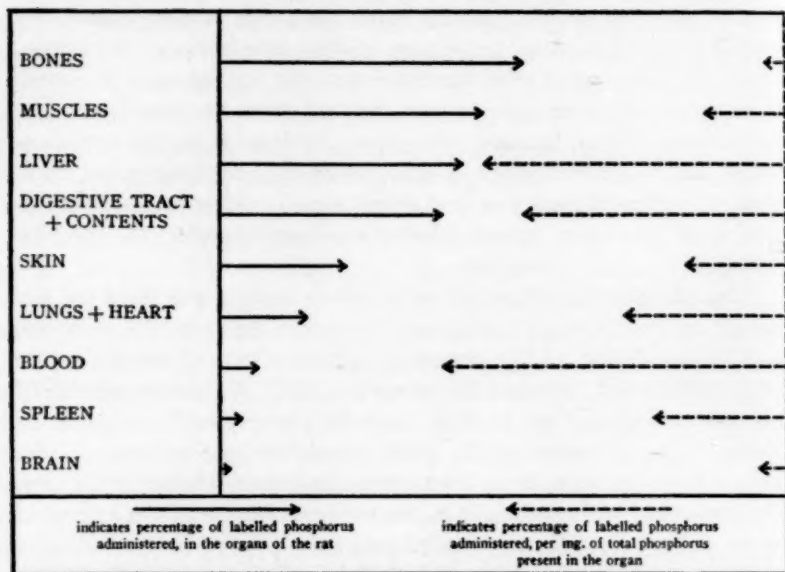


FIG. 1.—Labelled phosphoric content of the organs of a rat four hours after subcutaneous administration of labelled sodium phosphate (of infinitesimally small weight).

the cells of various organs. The loss through excretion is, in experiments of short duration, of no great significance. In experiments of four hours duration the skeleton and the muscles of a rat contain about the same amount of P^{32} , but in experiments of long duration we find the labelled phosphorus present in the body to be stored mainly in the skeleton. After the lapse of ninety-eight days, 92 per cent of the P^{32} present in the body is found in the skeleton (22). The retention of orally administered phosphate per unit weight of fresh tissue was found to decrease in the following order: bone, liver, gastrointestinal tract, heart, kidney, lungs, blood, muscle, skin, and brain (65).

The inorganic phosphorus of the plasma diffuses at a fairly fast rate into the corpuscles, and takes part inside the latter in the build-

ing up of acid-soluble organic phosphorus molecules, while nonactive molecules present in the corpuscles are decomposed, supplying inorganic phosphorus which diffuses into the plasma. Since, in experiments *in vivo* lasting four hours, the specific activity of the inorganic phosphorus and that of the acid-soluble organic phosphorus fractions extracted from the corpuscles does not differ much, it follows that most of the acid-soluble organic phosphorus molecules present in the corpuscles were renewed within that time. Labelled acid-soluble compounds, which were easily hydrolysed under the action of 1 *N* mineral acids, were found to be formed at a somewhat faster rate than nonhydrolysable ones. In experiments *in vitro* the rate of renewal of the acid-soluble organic compounds which are not easily hydrolysed (mainly composed of diphosphoglycerate) was found to be lower than in the first-mentioned case (23). Labelled hexosemonophosphate was found not to diffuse, or to diffuse at a much slower rate than the phosphate ion, into the corpuscles (14). The specific activity of the phosphorus of white cells of patients with leukemia was found to be lower than that extracted from red cells (92). Data are available on the specific activity of the blood phosphorus of human subjects compared to that of fetus and placenta (25), and the labelled phosphorus content of the ovaries of pregnant rabbits (26).

Skeleton.—Almost immediately after the introduction of active phosphate into the circulation an appreciable part of the P^{32} is to be found in the skeleton. The greatest part of the bone activity is due to the uptake of P^{32} activity by the apatite structure (8, 41, 42, 43, 47). As soon as five minutes after injecting labelled phosphate into the lymph sack of a frog, the tibia, freed of nonmineral constituents, is found to contain a tenth as much P^{32} as is present in the circulation (16). Early experiments lead to the results that the epiphysis phosphorus has a higher specific activity than the diaphysis phosphorus (43, 44, 45, 46). This was also shown by autodiagraphs taken by placing the cleaned bone on an x-ray film for some days (36). In the tibia of the rabbit, freed of nonmineral constituents, this ratio was found four hours after the start of the experiment to be about two. This difference was interpreted as due to better contact between the apatite structure and the lymph in the case of the soft bone.

The uptake of labelled phosphorus by the incisors and molars was investigated as well (43, 44). As the incisors of rats grow at a rapid rate, the newly formed tissue is bound to be labelled when the growth takes place in a labelled medium. But shortly after the administration of labelled phosphorus, P^{32} is found even in the tip of the in-

cisors, to which it must have been carried by the lymph circulation and where it has been incorporated into the apatite structure. After the lapse of eight days as much as 8 per cent of the administered labelled phosphorus is found in the incisors of rats. In the molars a less pronounced, but easily measurable, turnover takes place. In one molar of a human subject $1/300,000$ part of the labelled phosphorus given by mouth was found. A postmortal investigation of the bones and teeth of human subjects suffering from leukemia led to the result that the specific activity of 1 mg. of the tooth phosphorus amounts to only about a tenth of that of the rib phosphorus (93). Only a minute exchange is found in the enamel (95), which is highly mineralized and is, correspondingly, in exceedingly poor contact with the lymph containing the labelled phosphorus. The uptake of labelled phosphate by bones (25) and teeth placed in a phosphate solution was investigated. From varying concentrations of acid sodium phosphate in ethylene glycol the tissues were found to adsorb phosphate in accordance with Freundlich's adsorption isotherm (19).

While a very appreciable part of plasma activity enters the skeleton, only a small percentage of the skeleton phosphorus takes part in an exchange process in the course of four hours. This is at first sight a puzzling result which is due to the fact that the phosphate content of 1 gr. of fresh bone amounts to about 1,000 times that of 1 gr. of plasma. While $1/300$ part of the epiphysis phosphorus and $1/600$ part of the diaphysis phosphorus of the tibia of the rabbit is replaced within four hours by phosphorus atoms previously present in the plasma, the bulk of the bone phosphate of fully grown animals is only replaced at a very slow rate. In experiments on frogs at 20° the corresponding figures were found to be $1/360$ and $1/530$ (16).

The uptake of labelled phosphorus by rats with rickets was investigated as well (46, 67). The comparison of the uptake is made difficult by the fact that the phosphorus content of rachitic animals is appreciably lower than that of normal ones; thus the administered labelled phosphorus will be less diluted by nonlabelled phosphorus in the rachitic animals. A comparison of the ratio of the specific activity of the diaphysial phosphorus and the epiphysial phosphorus shows that this ratio is smaller in rachitic animals than in normal ones. It was found that the administration of vitamin D promotes the uptake of P^{32} by the bones (67), the uptake of inorganic phosphorus being raised by 25 to 50 per cent of its normal value. This result seems to support the view that under the influence of vitamin D a flow of some phosphate takes place from the muscles to the bones.

Acid-soluble organic phosphorus.—The formation of the organic phosphorus compounds which takes place inside the cells must be preceded by a diffusion of labelled phosphate from the plasma into the cells.² The rate of the latter differs much in different organs. The plasma phosphate was found to penetrate into the cortex of the kidneys at a rate fifty times higher than into the brain tissue (22). The most rapid turnover of the acid-soluble phosphorus compounds was found to take place in the kidneys and in the intestinal mucosa. In the course of four hours 36 per cent of the labile phosphorus of the adenylyltriposphate molecules present in the kidneys of rabbits was found to be replaced by labelled phosphorus, while the corresponding figure for the "hexose" fraction (100 min. hydrolysis in 1 N acid) was found to be 50. In the nonhydrolysed fraction the extent of turnover was 25 per cent. The corresponding figures in the fractions extracted from the liver were 55, 38, and 18, respectively (23). In contradistinction to the labile phosphorus of the adenosinetriphosphate molecule, the third stable phosphorus atom is labelled at a slow rate (34). In experiments on perfused cat liver it was found (27) that within one hour a large part of the inorganic phosphorus of the liver came into exchange equilibrium with the phosphate ions of the plasma. A high percentage of the adenylyltriposphate molecules and a minor percentage of the ester phosphorus were found in the course of one hour to be newly formed.

The replacement of the acid-soluble phosphorus of the muscle cell by plasma phosphorus was found to be a comparatively slow process. At 22° and 0°, 1 per cent and 0.3 per cent respectively of the acid-soluble phosphorus present in the muscle cells of frogs (exclusive of that which entered the extracellular space) was found to be replaced by plasma phosphorus in the course of three hours. In rabbits in the course of about twelve hours 10 per cent is replaced. As soon as the labelled phosphorus atoms entered the muscle cells they participated in a lively turnover, in which chiefly the easily hydrolysable, acid-soluble phosphorus compounds took part. Data are available on the turnover rate of the phosphorus compounds present in frogs (16, 35) and in rabbits (20, 22).

Study of intermediary reactions of glycolysis in vitro.—The labelled organic phosphorus compounds, hexosemonophosphoric and hexosediphosphoric acid, phosphoglyceric acid and adenylyltriposphoric

² The possibility that the synthesis of some of the compounds takes place on the surface of the cell walls facing the interspaces is not to be excluded.

acid (with two labelled phosphorus atoms) were prepared under the action of enzymes present in muscle juice or yeast (37, 38). The path which the phosphate radicals present in these molecules take in glycolytic (37) and other (14) processes was then investigated. Under the action of labelled adenylytriphosphate on Embden ester, Harden-Young ester is formed, which contains the labelled phosphorus in its labile phosphate group. When adding labelled adenylylphosphate to fresh muscle pulp, in which glycolysis takes place, no formation of active inorganic phosphate was found to occur. The adenylic acid thus did not transfer its phosphate groups under these conditions. When adenylylphosphate is formed from adenosine in the presence of hexosediphosphate and labelled inorganic phosphate in yeast, one-half of the phosphorus atoms present in the adenylylphosphate were found to be those originally located in the hexosediphosphate molecules while the other half were originally present as inorganic phosphate (37). The reaction: $2 \text{ triosephosphoric acid} + 2 \text{ pyruvic acid} + 1 \text{ adenosinemonophosphoric acid} + 2 \text{ phosphoric acid} \rightleftharpoons 2 \text{ phosphoglyceric acid} + 2 \text{ lactic acid} + 1 \text{ adenosinetriphosphoric acid}$, was investigated by using labelled phosphate (39). It was found that the labile phosphates of the adenosinetriphosphate molecule were entirely renewed within a few hours. This is also true when the above reaction runs from the right to the left. Experiments were made using much smaller amounts of enzymes than are present in an intact muscle but using about the same amount of adenosinetriphosphate. It was found that under these conditions it takes only 50 sec. for a labile phosphate radical present in the adenosinetriphosphate molecule to participate in an exchange process.

While transferring both hydrogen and labelled phosphate neither of the two stable phosphate radicals of the cozymase molecules were found to be replaced by active phosphate. A similar negative result was obtained when investigating the reaction $3\text{-phosphoglyceric acid} \rightarrow 2\text{-phosphoglyceric acid}$ in the presence of active phosphate and also that of the $\text{glucosemonophosphoric acid}$ into $\text{glucosehexophosphoric acid}$ in the presence of active phosphate. The ester fractions were found not to have taken up P^{32} . In the presence of active phosphate the reaction $2 \text{ phosphopyruvic acid} + 1 \text{ adenylic acid} = 2 \text{ pyruvic acid} + 1 \text{ adenosinetriphosphoric acid}$, was investigated as well. The phosphopyruvic acid was found to be entirely inactive, from which it follows that the above decomposition of phosphopyruvic acid is not a reversible one. This example illustrates the use of isotopic indicators in determining if and to what extent a reaction is reversible.

Protein phosphorus.—Little information is available on the turnover rate of phosphoproteins and nucleoproteins. The rate of formation of labelled casein in goats milk was investigated (18, 40); it was found that the formation of the casein molecule in the milk gland takes a few hours. The specific activity of the residual phosphorus found in the plasma and corpuscles of the blood was determined after removal of the phosphorus compounds which are soluble in acid and in ether-alcohol (14). Nucleoproteins were isolated from various organs of rabbits, by the method of Hammersten, twelve hours after the start of the experiment during which the activity of the plasma inorganic phosphorus was kept constant. The specific activity of the nucleoprotein phosphorus extracted from the liver, muscles, and thymus gland was found to be 4, 7.5, and 14.8 per cent respectively of the specific activity of the inorganic phosphorus extracted from the organ in question. A high turnover of the nucleoproteins thus takes place in the thymus gland (23).

Phospholipids.—Numerous investigations have been carried out on the formation of labelled phospholipids. In some of the early work it was found that even in the brain of fully grown animals a slow but clearly perceptible phosphatide turnover takes place (48). When tissues are grouped according to the value of the specific activity shown by their lipid phosphorus the following result is obtained (49, 50, 51): liver and intestine show high values; kidney, spleen, and lung moderate ones; heart, spleen, and bones low ones; central nervous system very low ones. These results confirmed and extended the information on the rate of turnover of the phospholipids in different organs obtained by using nonisotopic indicators as, for example, elaidic acid (71). To arrive at quantitative data as to the phospholipid turnover we have to compare the specific activity of the lipid phosphorus extracted from an organ with that of the inorganic phosphorus present in the cells of the organ in question. Experiments were carried out with rabbits, in which the specific activity of the plasma phosphate was kept constant (17, 20). About 10 per cent of the phospholipid of the intestinal mucosa, 7.4 per cent of that of the liver, 1.2 per cent of the gastrocnemius, and 0.9 per cent of the brain were found to be newly formed in the course of four hours. The above data relate to the lipid mixture which is soluble in petroleum ether. The objection has been raised to the determination of the turnover rate of phospholipids by the above-mentioned method, that the accumulation of labelled phospholipid in a given tissue may be the result of transfer from another tissue where it is synthesized. In experiments of appropriate dura-

tion, the specific activity of the liver phospholipids, for example, is much higher than that of the plasma phospholipids. If the labelled phospholipids of the liver are built up in another organ, they will have to pass through the circulation to reach the liver, and, in that case, would have to raise the specific activity of the plasma lipid to a value at least as high as shown by the phospholipid phosphorus of the liver. It is in considerations of this type that the idea of specific activity is found to be most useful.

The turnover of phospholipids was found to be higher in rachitic than in normal rats (46). Numerous data are available on the percentage of the activity of administered phosphate appearing as phospholipids in various organs at different times after administration (29, 30, 31, 32, 52, 53).

The curves demonstrating the change of the activity with time of the lipid phosphorus extracted from the liver of rats, reach a maximum after the lapse of about eight hours and decline rapidly after that time, while those showing the behaviour of the muscle lipid phosphorus show a slow rise from the start lasting for several days [data up to 150 hours are available (32)]. A progressive increase in the content of radioactive phospholipid was observed in the brains of rats for about 200 hours after the administration of labelled phosphorus (53). Since in these experiments the specific activity of the phosphorus atoms, which become incorporated into the newly formed phosphatide molecules, declines at a fast rate during the experiment, the above mentioned curves do not indicate the change in the amount of newly formed phosphatides with time but a more complex process.

In experiments taking nineteen to forty-three hours in which the specific activity of lecithin phosphorus extracted from the total body of rats was compared with that of the cephalin phosphorus, no difference was found. When comparing the fractions secured from the liver only, or from the intestinal mucosa, the lecithin fraction was found to be more active (55).

Should the phospholipid turnover in tumors be very high, and the release of labelled phospholipid into the blood be sufficiently fast, the investigation of the labelled phospholipid content of the blood of subjects suffering from cancer may be of diagnostic value. Investigations carried out from this viewpoint revealed, however, that the phospholipid turnover, determined four hours after subcutaneous injection of labelled phosphate, in both spontaneous and graft breast tumors of mice is intermediary between the turnover found in the liver and in the muscles of the same animal (21, 22). Liver and

muscle are typical representatives of tissues showing fast and slow turnover respectively. In a detailed investigation (32), determinations were made of the change in the activity of the phospholipid extracted from the cancerous tissue, and also from the liver and the muscles, during the course of 150 hours. The tumors investigated included mammary carcinoma, lymphoma, lymphosarcoma, and sarcoma 180. A uniform phospholipid activity was not shown by the different types of tumors examined, but on the whole it was found that the phospholipid turnover shows a greater resemblance to that of the more active tissues such as liver, kidney, and intestine, than to that of the less active tissues like muscle and brain. It is reported that the total phosphorus exchange per gram of tissue of the mouse as a whole is about the same for normal and lymphomatous animals. However, when individual tissues are examined there are differences (94).

Role of phospholipids in fat metabolism.—By making use of labelled phosphate important information was obtained on the role of phospholipid in fat metabolism (51, 30). It was found that in the intestinal mucosa of rats kept on an entirely fat-free diet an intense phospholipid metabolism takes place, and that the rate of the latter is increased by about half its value when the rats are fed with copious amounts of olive oil (about 20 gm. in the course of four days). As to the rate of formation of labelled phospholipid in the liver, the difference between rats kept on fat-free and on olive-oil diet is less, the increase in the formation amounting only to about 20 per cent, while no difference was found in the case of the muscles or the brain. Experiments were carried out (57) to determine whether or not the additional phospholipid molecules found in the blood after the administration of a fat meal were synthesized in the intestinal mucosa. Large amounts of labelled sodium phosphate and oil were fed to a dog. The additional phospholipid of the blood found a few hours after the start of the experiment was only slightly active, suggesting that the greater part of the additional blood phospholipid did not originate from the intestine but was possibly carried from the liver into the circulation. In experiments on perfused isolated cat liver (28), in which labelled phosphate was added to the perfusing lipemic blood, the specific activity of phosphorus secured from the phospholipids of the liver and also from the blood was found to be about twice as high as in corresponding experiments with normal blood. The formation of labelled phospholipids in slices of liver, kidney, or intestinal mucosa, was demonstrated by use of the Warburg apparatus (58) and such a formation was also found to take place in microscopic adrenal slices (26,

54). In the absence as well as in the presence of ingested fat the major part of the phospholipid turnover by the gastrointestinal tract can be ascribed to the small intestine, the stomach and large intestine playing but minor roles in this type of metabolism. That the turnover in the liver is not much influenced by processes going on simultaneously in the gastrointestinal tract was also shown in experiments in which the last mentioned tissues were removed (30). The influx of ingested fat increases the turnover of the liver phospholipids, however no difference in the turnover of the liver or kidney phospholipids was found in fasting animals which had been fed for several months on a fat-free diet and those which had been kept on a fatty diet. In these experiments (59) rats were fasted for twenty-four hours and then injected with labelled phosphorus subcutaneously, the animals being killed four hours later.

The rate of formation of labelled phospholipids in the liver of rats was found to be influenced by treating them with choline (31), cholesterol (33), or both. The phospholipid was found to show a high specific activity when the animals were treated with choline, though the difference did not exceed 40 per cent of the normal value and was usually only about 20 per cent. The change in the phospholipid activity begins about one hour after choline ingestion and its effect is greatly diminished ten hours later. On the other hand, the addition of cholesterol to the diet was found to diminish the phosphatide turnover in the liver of rats by about a sixth of its normal value. Such an effect was demonstrated as early as thirty hours after the feeding of cholesterol. When cholesterol and choline were both added to the normal butter diet, the turnover was found to be increased by about one-half of its normal value, while the total phospholipid content did not change much. These results are significant in view of the fact that fatty livers are readily produced by feeding rats diets rich in cholesterol and that excess lipid deposition in the liver under these conditions is prevented by choline. Betaine, like choline, accelerates the phosphorus lipid turnover in the liver in doses up to 50 mg. per rat; betaine is less effective than choline.

Egg and milk phospholipid.—The comparison of the specific activities of the lipid phosphorus extracted from different organs of the hen led to the conclusion that the bulk of the phosphatides incorporated in the yolks originates from the liver and is carried by the plasma through the ovary into the yolk. It was also found that as soon as the yolk left the ovary the formation of labelled lipids ceases (60). In contradistinction to experiments with rats (29, 51) and

rabbits (17), in which the phospholipid of the intestinal mucosa was found to show a higher specific activity than that of the liver, in laying hens, to which the labelled phosphate was administered by subcutaneous injection, the specific activity of the liver phospholipids was found to be decidedly higher than that of the intestinal mucosa (60). In the liver of laying hens, after the lapse of six hours, 1.8 per cent of the labelled phosphorus present was found as phospholipid phosphorus, while the corresponding figure for the gastrointestinal tract was 0.69 per cent. One hundred grams of blood of a laying hen contained 0.51 per cent of the labelled phosphorus as phospholipid phosphorus, the blood of the nonlaying bird contained only 0.13 per cent (52).

Labelled sodium phosphate was injected into hen eggs which were then incubated in some experiments for six, and in others for eleven, sixteen, and eighteen days. While the phosphatide phosphorus extracted from the embryo always showed a high specific activity, that extracted from the yolk was hardly active at all. The phosphatide molecules present in the embryo could not therefore have been taken from the yolk only, but must have been synthesized in the embryo. The investigation of the acid-soluble and residual (mainly nucleoprotein) phosphorus extracted from the embryo led to a similar result—namely, that the ratio, in which the labelled inorganic phosphorus atoms are incorporated into the different phosphorus compounds present in the embryo, is governed solely by probability considerations. Practically all the phosphorus atoms present in the various compounds of the embryo must pass through the stage of inorganic phosphorus; only the inorganic phosphorus present in the embryo is taken as such from the yolk or the white (70).

In contrast to the yolk phospholipid molecules, which are not formed to any significant extent in the ovary, a very substantial part of the phospholipid molecules present in the milk were found to be synthesized in the mammary gland (18, 40). This follows from the fact that the specific activity of the lipid phosphorus extracted from the mammary gland, and also from the milk itself, is higher than that obtained from the phosphatide of the plasma. The investigation of the activity of different milk fractions proved that no mixing occurs in the milk, while it is stored in the udder. It was also found that a few hours after the start of the experiment the specific activity of the phosphorus in the casein and in the acid-soluble organic phosphorus compounds is but slightly lower than that of the inorganic phosphorus in the milk. This makes it seem very probable that these sub-

stances are formed in the mammary gland from inorganic phosphorus. While most of the acid-soluble phosphorus compounds present in the corpuscles show a high turnover rate, the phosphatides present are only renewed slowly and incompletely (61, 63) within the lifetime of the corpuscle.

Exchange of phospholipid molecules.—The rate at which phosphatide molecules present in the plasma are replaced from the tissues was determined in the following manner (17): The plasma of a rabbit containing labelled phospholipids was injected into the veins of another rabbit, from which blood samples were taken at intervals. It was found that about one-half of the labelled phosphatide molecules left the plasma of the second rabbit within 1.5 hours. Of the organs, the liver took up most (32 per cent) of the phosphatide molecules which left the plasma; the muscles took up 3 per cent, the kidneys 1 per cent, and the brain 0.06 per cent in the course of four hours. In these experiments only an insignificant amount of labelled inorganic phosphorus was present in the circulation of the second rabbit, and therefore the presence of active lipid phosphorus in the liver, for example, of this rabbit was not due to any significant extent to the synthesis of labelled lipid, but to an exchange process going on between the plasma and the liver. The liver not only has a high phospholipid turnover, but its phospholipid molecules are easily replaced by those present in the plasma, suggesting an easy release of additional phosphatide molecules from the liver into the circulation. Experiments were also carried out (43, 62) in which an emulsion of radioactive phospholipid prepared from the livers of rats to which labelled phosphate had been administered, was injected into the femoral vein of another rat. An appreciable hydrolysis of the injected phospholipid was found to take place after the lapse of a few hours; the nonhydrolysed phospholipid was found mainly in the liver and the spleen. Such a labeled emulsion shows—as is to be expected—a different behaviour from that of plasma containing radioactive phospholipids formed *in situ* as in the experiment first discussed. As for the phospholipid molecules of the corpuscles, it was found (63) that a part of these exchange with those present in the plasma, but a great part of the phosphatide molecules present in the corpuscles remains unchanged for a very long time, possibly for the lifetime of the corpuscle.

Absorption and excretion of P^{32} .—The rate at which labelled sodium phosphate administered orally is absorbed depends a great deal upon the diet of the animal, especially its phosphorus and cal-

cium content.³ This explains the great differences in the extent of resorption found by different experimenters which vary, in experiments on rats, between 65 (47) and 98 per cent (51), values of 89 (20) and 90 per cent (42) being also recorded. Chicks retained 77 per cent and excreted 23 per cent in the sixty-day period (41); human subjects were found to excrete 28 per cent in the course of about eleven weeks (64). As to the labelled phosphate given by subcutaneous injection, 89 per cent was found to be absorbed in the course of a half hour by rats. Rabbits excreted within twenty-seven days 45 per cent of the P^{32} through the kidneys and 11.5 per cent through the intestine (45). The labelled phosphate content of the plasma of rabbits reaches its maximum after the lapse of about a half hour and declines to about a seventh of this value in the course of the next twenty-four hours (22).

The phosphorus present in the feces originates partly from nonabsorbed food and is partly of endogenous origin. The contribution from each source can easily be determined by making use of labelled phosphorus under strictly physiological conditions. An arbitrary amount of labelled phosphate is fed or injected and a comparison is made of the specific activity of phosphorus extracted from a feces and a urine sample which were collected after the lapse of several days or weeks. The ratio of specific activity of feces phosphorus to specific activity of urine phosphorus times 100 gives the percentage of phosphorus present in the feces which originates from the body proper (64). In experiments on human subjects the above percentage was found to be about 20 to 24. One-eighth of the P^{32} absorbed into the circulation of human subjects (8), and one-eleventh in the case of rats (47), was found to leave through the intestine. Phosphorus present in the normal diet is less efficiently absorbed than sodium phosphate, and in this connection it is emphasized that important information on the digestibility of different foodstuffs could be obtained by administering foodstuffs containing labelled phosphorus, for example, vegetables grown on a soil containing labelled phosphorus. No difference was found between rachitic and normal rats in the absorption or re-excretion of the labelled phosphorus in the gut (46). The absorption of phosphate by rachitic rats was found to be increased but little by vitamin D, the amounts absorbed being 73 and 83 per cent respectively (67).

³ The contribution of D. M. Greenberg to the *Annual Review of Biochemistry* (8, 269, 1938) contains a chapter on the developments in the study of mineral metabolism with the aid of isotopes as indicators.

Circulation in plants.—To determine if the phosphorus atoms present in the leaves and stem of a plant are bound to their places or freely migrate from one place to the other, maize plants and sunflowers are grown in a normal culture solution. After the lapse of a few weeks, when a number of leaves are formed, the plant is shifted to another culture solution containing labelled phosphorus. New leaves are formed which are found to contain labelled phosphorus, as they are formed in a culture solution containing such. The experiment shows, however, that also "old" leaves formed in nonactive medium contain very appreciable amounts of P^{32} . From this result it follows that a considerable exchange takes place between the phosphorus atoms present in the leaves and those present in the circulation stream. When cut leaves of sunflowers were placed for forty-eight hours in a labelled solution an uptake of a few per cent of the labelled phosphorus atoms into the cut leaf was found. Seeds were germinated until rootlets 2 to 3 cm. long were formed. Then the seeds were placed in small flasks with the rootlets dipping into a nutritive solution containing labelled phosphorus. The germs of maize seeds were found to contain an appreciable amount of P^{32} , the endosperm did not contain the slightest trace (66). The speed with which labelled phosphorus contained in a culture solution invades bean seedlings was measured and P^{32} was found to migrate 10 cm. per hour (68). It was concluded from experiments in which rooted cuttings of willow, geranium, *Sedum praealtum*, and *Bryophyllum calycinum* were used as plant material, that both the xylem and phloem function in upward transport of labelled phosphate and presumably of other ions (24). Experiments also have been carried out on the exchange of phosphorus in yeast (69).

THE ALKALI ELEMENTS

In experiments on human subjects (72, 73, 74) the radioactivity of the hand was used to indicate the rate of absorption of the ingested $Na^{24}Cl$ prepared by deuteron bombardment of sodium chloride (half life = 14.8 hours). Absorption of the radioactive sodium has been observed to begin within three to six minutes after the sodium chloride solution was taken by the fasting subject and to be completed three hours later. During a two-week period 10 per cent was found to be excreted. When $Na^{24}Cl$ was given by intravenous injection to rabbits (75), the $Na^{24}Cl$ in the blood was found to reach an almost constant level in about fifteen minutes (75). In an experi-

ment, in which plasma containing Na^{24}Cl was injected into another rabbit, a constant level was reached after the lapse of about three minutes (76).

Sodium is to be found mainly in the extracellular fluid and the amount of the latter was calculated by determining to what extent the radioactive sodium injected into the veins of rabbits became diluted in the body. A value amounting to 28 per cent of the body weight was found (75, 76). This figure may possibly be somewhat too high, as the mineral constituents of the skeleton were found to take up some Na^{24} (76). The problem if and to what extent the sodium present in the tissue is located in the cells, can be attacked by comparing the ratio of the sodium (Na^{23}) content of a plasma and a tissue sample with the ratio of their respective radioactive sodium (Na^{24}) content shortly after the injection of Na^{24} into the veins. While the extracellular sodium enters into exchange equilibrium with the injected sodium at a rapid rate, the penetration of sodium ions into the cells presumably will be a comparatively slow process. In the case, therefore, that the ratio of the Na^{23} and the Na^{24} content, respectively, of the plasma and the tissue is found to be the same, shortly after injecting the labelled sodium into the veins, we can conclude that all sodium present in the tissue is located in the extracellular space, and vice versa. Measurements carried out on rabbit gastrocnemius (76) lead to a value for the Na^{24} ratio which is in agreement with Na^{23} ratio found by Manery & Hastings (77), namely 11 per cent for the fat-free, blood-free tissue.

Na^{24} was found to penetrate easily into the corpuscles. Corpuscles of dogs were found to contain, after the lapse of seventeen hours, 0.56 times as much Na^{24} as plasma of the same weight. Experiments *in vitro* lead to similar values (78). In rabbit blood, after the lapse of twenty-four hours, the corresponding figure was found to be 0.25 (76).

With radioactive sodium as an indicator, the uptake and transportation of sodium chloride from the root as well as from the leaf were investigated together with its accumulation in the plant tissue (79). Cells of *Nitella* previously kept in labelled sodium chloride or labelled potassium chloride solution, respectively, were placed for different times in 0.01 M solutions of either lithium chloride, sodium chloride, rubidium chloride, or cesium chloride. It was then found that the rate of loss for labelled sodium was the most rapid in lithium solution, and slowest in that of cesium. In the case of labelled potassium, the rate of loss followed the Hofmeister series, rubidium ion was the

fastest, and lithium ion the slowest in replacing K^{42} . In distilled water the rate of loss of ions was very low (80).

Radioactive potassium (half life = 12.5 hours) is absorbed at a slower rate than radioactive sodium. While in the first half hour a fasting human subject absorbed 58 per cent of ingested $Na^{24}Cl$, the corresponding figure for $K^{42}Cl$ was 17 per cent (73). Fasting rats were found to absorb 90 per cent of $K^{42}Cl$ given by mouth in the course of half an hour (81). The absorption was found to occur mainly from the small intestine (82). Liver tissue shows an uptake which follows the absorption curve closely both in rise and fall. The K^{42} content of the skeletal muscle was found to increase for the first four hours and the excretion through the kidneys to take place at a nearly constant rate of about 6 to 7 per cent per day (82). Experiments on frogs and rabbits (76, 83) led to the result that twenty-four hours after the start of the experiment 1 gm. of fresh gastrocnemius tissue contains 1.35 and 1.45 times, respectively, more K^{42} than 1 gm. of plasma. This is about ten times as much as would be expected if all the K^{42} were present in the extracellular space of the muscle, but about twenty times less than one should find in case the total cellular potassium would exchange within twenty-four hours. From this and other results it follows that the bulk of the individual potassium ions present in the muscle cells do not leave these under normal physiological conditions. One gm. of fresh tibia diaphysis was found to contain after twenty-four hours as much K^{42} as 1 gm. of plasma. Corpuscles of rabbits were found to take up some K^{42} both in experiments *in vivo* and *in vitro*, contrary to the behaviour of the ions of sodium, the bulk of potassium ions present in the corpuscles was found not to exchange with the potassium ions present in the plasma (76).

An arrest of frog hearts takes place when the perfusing fluid lacks potassium. Attempts to restart the heart by adding $Na^{24}Cl$ or $K^{42}Cl$ of infinitesimal weight failed, while this could be effected by adding weighable quantities of ordinary potassium chloride. The action of potassium on the heart is thus not due to its radioactivity (91).

Radioactive potassium first accumulates in high concentration in the protoplasm of *Nitella* cells, and later diffuses into the sap (84, 85).

CARBON AND SULFUR

Though radioactive carbon obtained by bombarding boron with deuterons has a half life of only 21.5 minutes, it was successfully used

in the investigation of the assimilation of carbon dioxide by barley. After exposure to $C^{11}O_2$ for fifteen to seventy minutes, labelled carbohydrates and chlorophyll (as phytochlorin and phytorhodin) were isolated from leaves kept in complete darkness as well as from illuminated leaves. When, however, leaves were placed in the dark for about three hours prior to the administration of $C^{11}O_2$, the formation in the absence of light of labelled carbohydrates could not be detected. These results seem to indicate that the cell contains substances, either directly involved in photosynthesis or in respiration, which react with $C^{11}O_2$ reversibly in a nonphotochemical process (86).

Radioactive sulfur (half life = 86 days) is prepared by the bombardment of elementary sulfur. When 202 mg. were ingested as sodium sulfate by a human subject, 15 per cent appeared in the urine within the first nine hours, 32 per cent in the following fifteen hours. There was no detectable amount of radioactivity in the urine two days later (87).

When rats were fed with methionine containing radioactive sulfur cysteine containing labelled sulfur was found to be formed, while that was not the case when feeding labelled sodium sulfate. No exchange was found between the sulfide in cysteine and that in hydrogen sulfide (90).

HALOGENS AND IRON

The short half life of radioactive chlorine (37 minutes) hampers its usefulness. The rate of absorption of Cl^{38} by human subjects was found to differ only little from that of bromine (half life = 34 hours) and of sodium (73).

The I^{128} , which has a half life of 26 minutes, was obtained by irradiating ethyl iodide by slow neutrons emitted by a radium-beryllium mixture. Radioactive silver iodide, dissolved in a sodium thiosulfate solution, was injected into rabbits. About 0.2 per cent of the I^{128} was found, after the lapse of fifteen minutes, in the thyroid. In none of the tissues or fluids examined were quantities of iodine found which compared with that taken up by the thyroid (88).

Labelled iron (half life = 47 days), prepared by bombarding iron with deuterons, was found to be absorbed only in traces by a non-anemic dog; twenty-three hours after the last feeding, 0.3 per cent of the amount administered was found to be absorbed. Anemic dogs absorbed in a corresponding experiment 59 per cent of Fe^{58} . In the first-mentioned case 4 per cent, in the second 78 per cent of the amount absorbed was found to be present in the red blood cells. The

absorption was found to take place mainly in the small intestine. Three hours after the administration of 130 mg. of labelled iron as ferric sulfate, 1.06 per cent was found in the plasma and 0.23 per cent in the corpuscles; the liver contained 0.53 per cent, the spleen 0.12 per cent, and the marrow 2.0 per cent (89).

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NONPROTEOLYTIC ENZYMES

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HEMIN PROTEIDS WITH CATALYTIC ACTION

Keilin & Hartree (1) have made an excellent spectroscopic study of cytochrome oxidase prepared from heart musculature. This work revealed a hitherto unknown component, " a_3 ", which seems to be identical with Warburg's respiratory ferment or the cytochrome oxidase itself. In the reduced state, the α -band of the new component coincides with the cytochrome- a band at 605 m μ ; the γ -band coincides with the γ -band of cytochrome- a at 448 m μ . The new component, a_3 , could best be demonstrated in a carbon monoxide atmosphere, in which the band at 605 m μ broadens to 590 m μ , and the γ -band of the a_3 component is shifted to 432 m μ . The bands at 605 and 432 m μ are believed to be derived from a carbon monoxide compound of a_3 in the ferrous state. It is of interest to note that Warburg through photochemical experiments had already found the α -band of the carbon monoxide compound of the respiratory ferment to be in the neighborhood of 590 m μ and its γ -band in the neighborhood of 432 m μ . Keilin & Hartree also showed spectroscopically the existence of a hydrogen cyanide compound of a_3 .

Cytochrome oxidase ($\equiv a_3$) seems to be closely related to cytochrome- a , partly because the absorption spectra are so alike, and partly because they appear to give identical hemochromogens. The authors agree with Warburg that the hemin group is closely related to that present in chlorocruorin. The protein components of cytochrome- a and $-a_3$ also seem to be closely related. They are both bound to the cell structure and possess the same sensitivities against various denaturing factors. Certain peculiarities, however, still require explanation, as for example the failure of a_3 to be reduced anaerobically by cytochrome- c as would be expected. Neither could it be shown spectroscopically that the carbon monoxide compounds are light-sensitive.

Altschul, Abrams & Hogness (2) obtained a soluble cytochrome oxidase from yeast. It showed no sedimentation in one hour in a centrifugal field of 35,000 times gravity. Its activity was inhibited by hydrogen cyanide and carbon monoxide in the absence of light, and,

quite unexpectedly, by small amounts of catalase. These inhibiting relationships correspond so completely with the action of peroxidase on dihydroxymaleic acid as substrate (3, 4) that it would be interesting to know if this preparation shows any peroxidase activity.

Euler and co-workers (5) have postulated that a deficiency of cytochrome oxidase, cytochrome and possibly catalase is the cause of the abnormal metabolism in tumor tissue. It is now found that sarcoma tissue, free from cytochrome, contains even more catalase than muscle tissue. Stotz (6) found, by quantitative determinations of cytochrome oxidase and cytochrome-*c* in different rat tissues, that two sorts of rat tumors were to a practical extent free from cytochrome-*c*. These tissues cannot oxidize lactic acid—that is, they derive their energy from anaerobic glycolysis. However, other tumors were found to contain large amounts of cytochrome-*c*. Schultze (7) has found that copper in the diet is an essential factor for the formation of cytochrome oxidase. This had also been shown in 1934 by Cohen & Elvehjem (8) concerning cytochrome-*a*. In view of the close relationship between cytochrome oxidase and cytochrome-*a* pointed out by Keilin & Hartree (1), it is of course not at all surprising that copper is necessary for the formation of both. The cytochrome oxidases in plants have been studied by different authors (9, 10).

Cytochrome-c.—Previously, "pure" cytochrome-*c* preparations were thought to contain 0.34 per cent iron, but after a further purification by electrophoresis, the iron content was found to be 0.43 per cent. The purity of the cytochrome is verified by its homogeneous migration at all investigated pH values [Theorell & Åkesson (11)], so that the molecular weight may now be calculated as 13,000. The ferricytochrome shows, according to the pH of the solution, five different types of absorption spectra (11, 12), probably coinciding with different dissociation stages of the hemochromogen-forming groups. In very strong acid solutions, both of the hemochromogen-forming groups are freed from the iron atom, while in weaker acid solutions only one is released. The molal magnetic susceptibility in strong acid solution is 14,000, corresponding to five odd electrons, while in solutions with a pH higher than 4, it is 3,000, corresponding to one odd electron. Even if both the hemochromogen-forming groups are separated from the iron atom through the influence of strong acid, the hemin component remains bound to the protein component through the hemin's side chains. Concerning this linkage Theorell (13) could show that the porphyrin-*c* obtained through hydrolysis with hydro-

chloric acid is an addition compound of *l*-cysteine and porphyrin. The cysteine is bound to the side chains in the 2- and 4-positions by means of the sulfur in the thioether linkages. However, it appeared possible later that the thioether linkages are not found preformed as such, but arise during hydrolysis (14). Zeile & Meyer (15, 16), through hydrolysis under conditions which should exclude the possibility of the formation of thioether linkages during the hydrolysis, still obtained a porphyrin-*c* preparation which was identified as dicysteine porphyrin. It is therefore probable that the type of linkage assumed by Theorell (13) actually exists in the cytochrome. However, it still remains to be explained that on hydrolysis with hydrochloric acid for a short time, a preparation is obtained with fewer than two atoms of sulfur per molecule of porphyrin. It is also noteworthy that a preparation with the optical properties of the *c*-hemin could be obtained from oxyhemoglobin after long treatment with dilute hydrochloric acid [Schales (17)].

Quantitative methods for the determination of cytochrome-*c* have been presented by Hogness *et al.* (19) and Fujita *et al.* (20), and have been used in widespread studies of the content of cytochrome-*c* in different animal organs. These investigations show that the cytochrome-*c* content varies so widely from organ to organ that one must question whether, in all these cases, the cell respiration proceeds through cytochrome-*c*. Actually this relation has been shown only for yeast (Haas, 21). According to Potter & Lockhart (22) both cytochrome-*b* and -*c* are necessary as intermediate carriers for the oxidation of both dihydrocozymase and succinic acid by molecular oxygen.

The spreading of cytochrome into monomolecular layers has been studied (18).

Catalases.—It is now definitely established that the liver catalase from cattle, crystallized according to Sumner *et al.* (23), gives an activity value of Kat. f. = 30,000,¹ while the pure catalase from horse liver possesses an activity which is twice as large [Agner (24, 25)]. Sumner & Dounce (23) have presented some modifications in the method of preparation for beef-liver catalase, and Dounce & Framp-ton (26) obtained crystals of horse-liver catalase, whose activity and degree of purity, however, were somewhat lower than for Agner's

¹ The recently published value of 38,000 obtained by Sumner & Dounce is based on an incorrect interpretation of Agner's determinations.

amorphous preparation. Up to the present it is only possible to remove all iron impurities through electrophoresis; using this method, Agner could show that the total iron content of horse-liver catalase, 0.09 per cent, corresponds to the content of protohemin. Besides the hemin, there is also present in the prosthetic group of the catalase a blue dyestuff, which is maintained by several authors to be a verdo-hemochromogen. Lemberg *et al.* (27) have lately isolated biliverdin in a pure state from this blue fraction, but they assume it to be present in the enzyme in the form of the complex iron compound, bile-pigment hematin. However, according to Agner, all the iron may be shown to be present in the protohemin fraction, so that Lemberg's view can scarcely be correct. Lemberg *et al.* found the molecular relation between biliverdin and protohemin in horse-liver catalase to be 1:3, and therefore assume that every catalase molecule contains one molecule of biliverdin and three molecules of hemin. However, it would seem more likely from Agner's analyses that every molecule contains four molecules of hemin. Cow-liver catalase contains less hemin; according to Sumner & Dounce, there are perhaps two hemin molecules per molecule of enzyme. However, the cow-liver catalase can evidently not be freed from iron impurities by crystallization and its composition is not completely clear. The function of biliverdin is entirely unknown. The copper discovered by Agner can be removed without loss of activity (23, 25).

The proposed theory of Keilin & Hartree (28) for the function of catalase, according to which the hemin iron is reduced by hydrogen peroxide and reoxidized by oxygen, has been criticized by Johnson & Schouwenburg (29) and by Weiss & Weil-Malherbe (30). Keilin & Hartree defend their theory against these criticisms (31, 32).

The immunochemistry of catalases has been studied by Tria (33) and by Campbell & Fourt (34). Kleinzeller & Werner (35) have found an increasing catalase activity in the tissues of chick embryos from the fourth to the fifteenth day of development while, at the same time, according to Warburg *et al.* (36), there is a decrease in the anaerobic glycolysis activity. Louvier & Serfaty (37) have demonstrated certain characteristic differences in the changes of catalase activity in the blood of hens and roosters during development.

Peroxidase and dioxymaleic acid oxidases.—A definite purification of peroxidase has not yet been achieved, in spite of the fact that this enzyme was one of the first subjected to serious attempts at puri-

fication more than twenty years ago by Willstätter and his co-workers. Unanimity of thought now prevails concerning peroxidase, which is probably a hemin proteid resembling methemoglobin [Keilin & Mann (38)] to a certain extent. The function of peroxidase has been assumed to lie in the oxidation of polyphenols, not by molecular oxygen, but by hydrogen peroxide. The hydrogen peroxide has been supposed to arise in one or another biological process.

Theorell & Swedin (4) have discovered a new function for peroxidase through their investigations with dihydroxymaleic acid oxidase. It proved to be impossible, preparatively, to separate the latter from peroxidase. Even the purest peroxidase which could be obtained exhibited a strong and constant aerobic oxidase activity on dihydroxymaleic acid. The conclusion reached was that peroxidase and dihydroxymaleic acid oxidase are very likely identical, although several factors apparently weigh against this conclusion. Thus, for example, there exist meaningful differences in inhibition by hydrogen cyanide, copper salts, fluoride, azide, carbon monoxide, etc., when the peroxidase activity in the purpurogallin test is compared with the oxidase activity on dihydroxymaleic acid. These discrepancies, however, very likely only depend on different reaction conditions for the enzyme iron in the two reactions. Carbon monoxide does not at all inhibit in the purpurogallin test, but produces a light-sensitive inhibition in the dihydroxymaleic acid test. Spectroscopic observation of the enzyme in the presence of dihydroxymaleic acid and of different inhibiting agents disclosed that those substances that inhibit dihydroxymaleic acid oxidase activity also produce typical changes in the observed peroxidase spectrum. Concerning peroxidase in respect to its behavior with dihydroxymaleic acid, small amounts of hydrogen peroxide are formed and subsequently take part in the reaction. Thus the difference between this and an ordinary peroxidase reaction is that the hydrogen peroxide is here formed from the substrate itself. Removal of the hydrogen peroxide from the reaction mixture by the addition of catalase halts the reaction. It is clear that if dihydroxymaleic acid is present in the tissues of plants, the peroxidase can act as an aerobic oxidase. According to Gay & Genevois (39) and Gatet (40) dihydroxymaleic acid can be found in grape juice. Possibly the dihydroxymaleic acid acts as an intermediate catalyst since its oxidation product, the corresponding diketo acid, can easily be reduced again to dihydroxymaleic acid [Banga & Philippot (41)].

DIFFERENT METAL PROTEIDS AND METAL CATALYSTS

Animal and vegetable tissues contain many different metals in varying amounts. It has long been known that many of these, even if present only in small amounts, are indispensable to the correct maintenance of vital processes. Their mechanism of action, with the exception of iron, has been to a large degree unknown until quite recently. However, it had been realized that several of them, if not all, should be able to enter into the structure of proteids with enzymatic properties. This hypothesis has now been clearly verified in the case of copper, zinc and manganese and there is little doubt that analogous relationships will be shown for several other metals. A short survey of last year's experiments concerning catalytic metal activities may therefore be useful.

As to whether ions can enter into enzymes without the mediation of porphyrin remains unknown. Davidson (42, 43) has found a significant amount of iron in purified uricase, but Holmberg (44, 45) has succeeded in removing most of the iron without a loss of activity. After Kubowitz's (46) purification of the copper proteid, polyphenol oxidase, Keilin & Mann purified two catalytically active copper proteids, the polyphenol oxidase from mushrooms (47) and the long-known laccase (48). The mechanism for the activity of polyphenol oxidase on catechol has been investigated by Jackson (49). It is still not known which of the metals enters into tyrosinase as the active group [Gould (50), Califano & Kertész (51)].

The so-called ascorbic acid oxidase is probably a copper proteid, but one must doubt whether we are dealing with any well-defined enzyme. On the contrary, it is felt that a certain ascorbic acid oxidase activity results when copper ions unite even with unspecific proteins (52). Ebihara (53, 54) has purified to some degree the ascorbic acid oxidase from cucumber and has studied its properties. The question of whether copper porphyrins possess similar catalytic activities to the iron porphyrins has been examined by Salomon (55), who found, however, that their properties render them unsuitable as biological catalysts. Manganese takes part in an even greater number of biological processes. It is said to enter into arginase as the prosthetic group (56). Together with cytochrome-*c* it gives a dihydroxymaleic acid oxidase activity (3), which depends upon autoxidation of the substrate by the manganese ion and the formation of hydrogen peroxide. The manganese can be replaced, therefore, by a small amount of hydrogen peroxide added at the beginning (4).

Rudra (57) has shown that manganese probably plays a part in the synthesis of ascorbic acid in the liver. The author finds an increased production of ascorbic acid by the addition of manganese ion to liver tissue incubated with galactose. Man and the guinea pig, which cannot synthesize ascorbic acid, show such a low manganese content in the liver that this deficiency is probably the cause of the poor synthetic power (58). Manganese is supposed to be the coenzyme of a proteid which is important in the synthesis (59).

A deficiency of manganese in the diet of rats brings about a peculiar symptom complex, which can also be produced by an overdosage with thiamin (60). The overdose of thiamin appears to exhaust the organism's supply of manganese. In accordance with this, the thiamin-overdosage symptoms disappear on the addition of manganese to the diet.

Brückmann & Zondek (61) have carried out extensive determinations of iron, copper and manganese in human organs.

Manganese can also alternate with magnesium in certain phosphate interchanges and in the oxidative decarboxylation of pyruvic acid (62, 63, 64).

According to Bernheim & Bernheim (65) vanadium, as a prosthetic group, enters into a liver proteid which catalyzes the aerobic oxidation of phospholipids. Manganese and, to a lesser degree, cobalt, inhibit this process (66), while nickel, iron, titanium, and chromium are inactive. Titanium in the form of sodium pertitanate inhibits the rapidly occurring oxidation of cysteine to cysteic acid in ground liver, but not the oxidation to cystine. Titanium thus exerts a regulating activity on the oxidation of sulfhydryl compounds (67). According to Medes (68), the enzyme that oxidizes cysteine to free sulfate via cysteine sulfinic acid and cysteic acid can be found in the mother liquor after the isoelectric precipitation of cytochrome oxidase from liver.

According to Keilin & Mann (69), zinc forms the prosthetic group in the carbonic anhydrase from red blood cells. The enzyme catalyzes the breakdown of carbonic acid to carbon dioxide and water. The zinc content in the best preparations was 0.34 per cent. No other metals could be found present.

Lohmann & Kossel (70) have shown that zinc either inhibits or stimulates yeast decarboxylase according to the concentration of the zinc.

Horecker *et al.* (71) hold that aluminum ions exert a promoting

effect on the succinodehydrogenase-cytochrome system. The same applies to chromium and the rare earths, but in contrast to aluminum these could not be shown to be present in the preparation. It would seem possible that aluminum also takes part in cell oxidation processes.

According to Mendel *et al.* (72), magnesium and calcium stimulate the activity of choline esterase, while potassium accomplishes the direct opposite. Thus magnesium and calcium accelerate and potassium inhibits the destruction of acetylcholine. This situation would seem to explain the influence, long since noted, of these ions on nervous irritability. The enzyme in horse serum that splits O-acetylthiamin is probably identical with choline esterase (73, 74, 75).

FLAVOPROTEINS

Certain progress, achieved during 1938, in our knowledge of the flavin-adenine-dinucleotides should first be noted.

To begin with it may be mentioned that Weygand & Birkofer (76) presented a modified method for purifying the "old" yellow ferment based on the adsorption of Warburg & Christian's product on aluminum hydroxide-Cy, with subsequent elution with ammonium sulfate solution at pH 8.2. With this procedure a preparation with the same flavin content as Theorell's (77) was obtained. It was free from the fumaric hydrase discovered by F. G. Fischer. A reversible splitting of the ferment was attained in 0.01 — 0.02 *N* HCl at 0° by chromatographic absorption on Frankonite by which the prosthetic group is adsorbed. The acid protein solution may be reactivated to an appreciable extent by dialysis against water. The yield is approximately the same as that obtained by Theorell's earlier method.

Flavin-adenine-dinucleotides and d-amino acid oxidase.—Abraham (78, 79) has shown that through a mild alkaline hydrolysis of Warburg & Christian's dinucleotide, cophosphorylase (adenosine-5-phosphoric acid) is formed in amounts up to 40 per cent. In this respect the flavin dinucleotide behaves similarly to diphosphopyridine nucleotide but oppositely from triphosphopyridine nucleotide. Furthermore, flavinphosphate is formed but not free flavin. Obviously, therefore, the flavin-adenine-dinucleotide is made up through a union of flavin phosphoric acid with adenosine-5-phosphoric acid.

Negelein & Brömel (80) describe extensively the purification of the protein component of *d*-amino acid oxidase. Since this ferment is comparatively strongly dissociated into protein and prosthetic group,

these components must be prepared separately. On the assumption that the "purest" protein preparation was actually pure and that it reacted with the dinucleotide in the molar ratio of 1:1, the molecular weight of the protein could be calculated as 100,000. However, for the sake of analogy it was assumed that the degree of purity was more nearly 0.7, thus giving the enzyme a molecular weight of 70,000.

Dihydroflavin-adenine-dinucleotide seems to combine with the protein of amino acid oxidase to form a practically undissociated compound. Upon coupling with the protein, the light absorption bands of the dinucleotide are shifted somewhat to the "long-wave" side in the same way as the "old" yellow ferment.

In tissues from rats which were kept on a riboflavin-poor diet, the amount of *d*-amino acid oxidase (81) was diminished. Klein (82) has found that feeding with thyroid tissue increases the *d*-amino acid oxidase content of rat liver.

The activity of the enzyme with various amino acids as substrates has been investigated by Felix & Zorn (83), unfortunately with an impure enzyme preparation. Krebs (84) has examined its activity on *d*(+)-prolin. The oxidative deamination of *d*(—)-alanine to ammonium pyruvate is said to be reversible (85, 86).

Wakabayashi (87) has studied the deaminases of ribo-adenylic acid occurring in animal tissues. Adenylic acid deaminases were separated from adenosine deaminases. Adenine deaminases do not seem to be present in animal tissues. Borsook & Dubnoff (88) have extracted from *Aspergillus Wentii* a specific enzyme that deaminizes free and combined adenylic acid. Its chemical nature is still entirely unknown.

Xanthine oxidase.—Ball (89) has now described more completely the purification and properties of xanthine oxidase from milk. The product has a strong gold-brown color. The light absorption curve shows a broad maximum between 400 and 500 m μ , the shape of which differs, however, from that of the known flavin compounds. If the ferment is reduced through the addition of hypoxanthine under anaerobic conditions, only a part of the color disappears. The difference in light absorption suggests that this is a flavin compound which is reduced. Upon the addition of sodium hydrosulfite the brownish color which remains after the hypoxanthine treatment disappears still further. When methanol is added to a solution of the ferment a white precipitate is obtained and flavin-adenine-dinucleotide can be shown to be present in the filtrate. However, the prosthetic group is not composed solely of the dinucleotide. A reversible splitting could be

obtained by dialyzing the ferment against running water for two weeks, which inactivated the inner fluid. This, however, regained its activity on the addition of the outer liquid, but not on the addition of flavin-adenine-dinucleotide. Ball suggests that the enzyme has two active groups, since the activity in electrophoretic experiments follows in parallel the migration of both the flavin and the brown component. Assuming that the best enzyme preparations were pure and contained one molecule of flavin per molecule of enzyme the molecular weight should be 74,000 [cf. Philpot (93)].

Hydrogen cyanide inactivates the ferment irreversibly according to the work of Szent-György in 1926. Ball holds that this effect is due to destruction of the protein component.

It seems remarkable that the enzymatic oxidation of hypoxanthine does not form uric acid, for the addition of uricase after the reaction had run to completion resulted not in oxygen consumption, but, instead, in the production of an unknown gas. In these experiments Ball used acetaldehyde or hypoxanthine as substrates, but not dihydrocozymase. However, Ball states that the flavoprotein from milk, described by Corran & Green (90), may be derived from xanthine oxidase which through their treatment in strong acid (pH 4) would be inactivated with respect to aldehyde and hypoxanthine activity, but would remain active towards dihydrocozymase. This assumption is supported as well by Corran *et al.* (91), as by Ball & Ramsdell (92). The former have, independently of Ball, come to the same conclusion as he. The group which is responsible for the aldehyde, xanthine, and hypoxanthine activity is very sensitive and its activity diminishes continuously during the preparation (most slowly at low temperatures and high salt concentrations), while the dihydrocozymase activity remains constant. Drying, or treatment with lead acetate, according to Corran & Green's earlier method, likewise destroys this sensitive group. The use of methylene blue in place of oxygen as the reoxidizing agent in the activity tests with aldehyde, xanthine, and hypoxanthine was found to be satisfactory. A closer investigation of the preparation showed that only 35 per cent of the light absorption at 450 m μ depended upon the flavin component, while the addition of sodium hydrosulfite decreased the light absorption by 60 per cent. Thus, even the brownish component bleaches somewhat with the strong reduction (cf. 89).

The activities toward hypoxanthine, acetaldehyde, and dihydrocozymase are of the same order of magnitude as the respective turn-

over-numbers, viz, 306, 570, and 260 per min. Strangely, none of the substrates can rapidly reduce the flavin component anaerobically. A small part is possibly reduced quickly, but the complete reaction goes slowly. For this reason it is doubtful whether the flavin component is of any importance for the activity. Analyses of the purest preparations in the ultracentrifuge (93) showed a marked degree of heterogeneity. The molecular weight of the principal component was 220,000 to 320,000 which should give 1.4 to 3.1 flavin molecules per molecule of protein.

Blanck *et al.* (94) have studied the xanthine oxidase from rat blood; it was found to have properties similar to that from milk. Subrahmanyam *et al.* (95) have obtained a flavoprotein preparation from liver which also resembles xanthine oxidase.

Coenzyme factor, diaphorase.—Through treatment of a cytochrome oxidase preparation of heart muscle with alcoholic ammonium sulfate solutions and by other purifying operations, Straub (96, 97) isolated a flavoprotein with 0.65 per cent flavinphosphate in the prosthetic group. When this was split off by heating it showed itself able to function as the prosthetic group in *d*-amino acid oxidase and is therefore probably identical with flavin-adenine-dinucleotide.

After inactivation of the primary protein component by heating, an excess of protein of the *d*-amino acid oxidase is added, after which the activity is tested with *d*-alanine as substrate. The process possesses the main disadvantage that no knowledge may be gained of the activity of the primary protein component.

The isolation and treatment of the enzyme is facilitated by its unusually great heat stability. The percentage of riboflavin in the purest preparations is 0.54 per cent or, calculated as flavinphosphate, 0.66 per cent. Thus, for one mole of flavin there are 70,000 grams of protein. A noteworthy difference from the earlier known flavoproteins is that Straub's proteid fluoresces like free flavin. From this it is clear that the linkage between the flavin-adenine-nucleotide and the protein must be of another type than in *d*-amino acid oxidase.

In the case of Straub's proteid it is definitely established that the activity results from oxidation and reduction of the flavin component (98, 99). According to Corran, Green & Straub (100), the proteid is reduced by dihydrocozymase (Co_1H_2) and also, supposedly, by Warburg & Christian's dihydrocoenzyme (Co_{11}H_2). It is slowly reoxidized by oxygen, and rapidly reoxidized by methylene blue. The physiological reoxidation probably occurs through the cytochrome

system. There is little doubt that Straub's proteid is identical with Euler and co-workers' diaphorase and Green and co-workers' coenzyme factor, because the activity and amount of the flavoprotein are sufficiently high to account for all the diaphorase activity. Other agreements are also pointed out. Before the purification of the enzyme it was considered that this ferment could not be a flavoprotein because even the moderately active solutions were uncolored. They might have been expected to show a yellow color if the turn-over-number had been of the same order of magnitude as that of the "old" yellow ferment. However, Straub's pure flavoprotein showed itself to have a turn-over-number 100 to 200 times larger than that of the "old" yellow ferment (8,000 per min. in place of 50). Even a solution without noticeable color can therefore possess great catalytic activity.

Adler, Euler *et al.* (101, 102, 103) have found that there supposedly exists diaphorases for both Co_1H_2 and Co_{11}H_2 , for the relationship between the activity of the one and of the other varies considerably in extracts from different organs. Lockhart (104) and Potter (105) have published some observations on this problem. According to Haas, Horecker & Hogness (106), there can be isolated from yeast a colorless factor which catalyzes the reduction of ferri-cytochrome-*c*. One μg . of this catalyst possessed the same activity as 160 μg . of the "old" yellow ferment in Theorell's experiment (107).

Their conclusion that the new enzyme could not be a flavoprotein may be premature, since in Theorell's experiment the yellow ferment was used in large excess, as was not the case in the experiments of Haas *et al.* Also, it is possible that it is a flavoprotein with a high turn-over-number as in the case of diaphorase. Stern & Melnick (108) found that if a phosphate extract from heart muscle is centrifuged with such a speed that cytochrome oxidase (containing cytochrome-*a*, -*b*, and -*c* and also succinodehydrogenase sediments out, there remains in solution a catalyst necessary for the oxidation of succinic acid. The relation of this factor to diaphorase is still not clear.

Fumaric acid hydrogenase.—Preparations of the "old" yellow ferment usually contain a factor which hydrogenates fumaric acid and which can be split off from the enzyme by electrophoresis (109). Supposedly, the prosthetic group is not riboflavinphosphate but flavin-adenine-dinucleotide. Strangely enough, after the enzyme is split by ammonium sulfate in acid solution, the protein and the prosthetic group will recombine slowly. Like *d*-amino acid oxidase, the enzyme is moderately strongly dissociated into protein and prosthetic group.

The turn-over-number is high—of the order of 3,000 mols of hydrogen per minute, per molecule of enzyme.

PYRIDINE-NUCLEOTIDE PROTEIDS

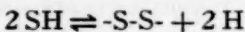
The most important result in this field has been achieved by Warburg and Christian (110) who have isolated the protein of the "*oxydierendes Gärungsferment*" from yeast in crystalline form. The method of preparation is of interest in one respect: ammonium sulfate is not used for fractionating the Lebedew juice proteins; instead, nucleic acid at different hydrogen ion concentrations is employed. Like the protein isolated by Negelein & Wulff (111) in 1937, the new protein forms a dissociating proteid with cozymase. According to Warburg's view both of the now crystallized proteins are the only ones that take part in the pyridine catalysis of fermentation, because with both of these proteins in the presence of cozymase there can be obtained the complete oxidation-reduction cycle of the pyridine nucleus, occurring during fermentation. The protein components closely resemble each other to a striking degree in respect to elementary composition, proteid dissociation constants, and turn-over-numbers. While the reducing enzyme catalyzes the reversible reaction: acetaldehyde + dihydropyridine \rightleftharpoons alcohol + pyridine, the oxidizing enzyme catalyzes the reversible reaction: 3 phosphoglyceric aldehyde + pyridine + H₂O \rightleftharpoons 3 phosphoglyceric acid + dihydropyridine. However, Negelein & Brömel (112) have found that the latter reaction is somewhat more complicated in that the Fischer ester (phosphoglyceric aldehyde) is first esterified by phosphoric acid to 1,3-diphosphoglyceric aldehyde which is reversibly transformed by the new oxidizing enzyme to 1,3-diphosphoglyceric acid. The equilibrium in this reaction can be effected both by the hexokinase and isomerase enzymes and by the phosphorylating adenylic acid system. This does not mean, however, that the hydrogenation of the pyridine nucleotides in the fermentation is thermodynamically linked to the phosphate esterification occurring through the adenylic acid system. This has been pointed out by Warburg & Christian in respect to the theory developed by Meyerhof and co-workers (113).

While the chemical constitution of the diphosphopyridine-nucleotide may now be regarded as fairly well established, this is not the case with the triphosphopyridine-nucleotide. Through alkaline hydrolysis under proper conditions, adenosine-5-phosphoric acid was obtained from "Co_I," but not from "Co_{II}." The third phosphoric acid

molecule of "Co_{II}" is split off only with difficulty on hydrolysis (114). Concerning the biological synthesis of the pyridine dinucleotides from nicotinic acid amide, Vilter *et al.* (115) find, in opposition to Kohn & Klein (116), that normal erythrocytes on incubation with nicotinic acid amide do not synthesize nucleotides; on the other hand leucocytes from leucemic blood give a marked synthesis.

DEHYDROGENASES

Succinodehydrogenase can be prepared from hog heart, free to a large extent from diaphorase (117) and fumarase (118). It has been generally accepted that succinodehydrogenase reacts directly with the cytochrome system, but according to experiments by Hopkins, Lutwak-Mann & Morgan (119), it would seem now that an intermediate enzyme must exist, linked between them. It can be separated, preparatively, from succinodehydrogenase by extraction with bile salts in aqueous solution. The chemical nature of the intermediate catalyst is still entirely unknown. It can not be replaced by any of the known coenzymes nor by glutathione, thiamin, epinephrine, ascorbic acid, or nicotinic acid amide. Euler & Hellström (120) and Cedrangolo & Adler (121) conducted a number of additional experiments which support the theory that an oxidation-reduction reaction of the character



is responsible for the activity of the succinodehydrogenase. Horecker (122) shows that citrate, oxalate and malonate in proper concentrations are able to stimulate succinodehydrogenase. The explanation of the stimulation is that traces of copper impurities are so bound that their inhibiting activity disappears. The inhibition of succinodehydrogenase by copper salts (123, 124) has been stated to be dependent upon the binding of the "activity-necessary" sulfhydryl groups. However, this interpretation is not certain since an inhibition by copper can be accounted for by other factors, as in the case of the dihydroxy-maleic acid oxidase (4).

Lacticodehydrogenase from yeast also seems to require a hitherto unknown intermediate catalyst to react with the cytochrome system (125).

E. coli contains, according to older, differing statements, an *l*-malic acid dehydrogenase which can be extracted (126). Cozymase and diaphorase bring about the reduction of methylene blue. *Clostridium butylicum* forms and secretes an enzyme which racemizes *d*- and *l*-

lactic acid (127). The enzyme is inhibited by 0.05 *M* HCN. In view of the high concentration of hydrogen cyanide, the conclusion that the enzyme is an iron or copper proteid may be doubted. Formic dehydrogenase from *E. coli* (128) is similarly inhibited by cyanide in high concentrations.

Citric-dehydrogenase does not seem to exist. According to work by Martius (129), Adler and co-workers (130) and Johnson (131), the enzyme aconitase establishes an equilibrium between citric acid, cis-aconitic acid, and isocitric acid. It is the isocitric acid which is dehydrogenated by a specific dehydrogenase containing Co_{II} as the prosthetic group (132, 133). The aconitase is not identical with fumarase (134, 135, 136). It is widely distributed in both animal and vegetable tissues. At equilibrium, at pH 7.4, 80 per cent of citric acid, 4 per cent of aconitic acid, and 16 per cent of isocitric acid are formed (131). Isocitric acid dehydrogenase is activated in the presence of Co_{II} , manganese, and magnesium (132).

Lang (137, 138) has found an enzyme in liver and muscle which dehydrogenates saturated fatty acids, particularly the higher homologues. Unsaturated fatty acids are not attacked, or, if so, only with difficulty. For example, oleic acid is formed from stearic acid and is not further affected. The dehydrogenation thus takes place in the center of the molecule. Consequently, this type of dehydrogenation is unrelated to Knoop's β -oxidation. Higher unsaturated fatty acids can not be formed with the help of this new enzyme system, nor probably by any enzyme system, for it is known that these acids are necessary nutritional factors. The pH optimum of the enzyme is 8.0. It is inhibited by hydrogen cyanide but not by iodoacetate or fluoride. It requires a coenzyme for its activity and this could be identified as muscle adenylic acid.

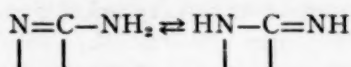
Kritzmann (139) has extracted an enzyme from muscle, aspartic acid transaminase, which is like the glutamic acid transaminase but less stable. The transaminases contain a thermostable coenzyme of unknown nature.

THIAMIN-PYROPHOSPHATE PROTEIDS

In this sphere there is a striking lack of balance in our knowledge of the prosthetic group, thiamin-pyrophosphate, and the protein part of the holo-enzyme, decarboxylase. While a continually growing amount of literature deals with thiamin and thiamin-pyrophosphate, we know almost nothing of the specific proteins for thiamin-pyrophos-

phate. Most of the published works in this connection fall outside the limits of this chapter.

Concerning the mechanism for the action of thiamin-pyrophosphate, it may now be clearly stated that an oxidation-reduction occurs as in the case of the pyridine nucleotides. Peters and co-workers (140) support this view of Lipmann. Stern & Melnick (141) have found, through reduction experiments on thiamin and cocarboxylase with platinum or palladium plus hydrogen, that the products obtained are not autoxidizable dihydro compounds. In view of this, it is highly credible that an oxidation-reduction enters into the activity mechanism, for dihydrothiamin was found to be inactive in the test system of the authors (supposedly because it cannot be phosphorylated), while dihydrococarboxylase proved to have the same activity as cocarboxylase in these experiments. The pyrophosphate group is assumed to serve the same purpose as the phosphoric acid groups in the flavin and pyridine nucleotides—namely to form a couple with a specific protein—and thus to make possible the rapid and reversible oxidation-reduction of the thiamin nucleus. Langenbeck had earlier predicted on the grounds of model experiments that the decarboxylase enzyme, when once purified, would be found to possess a primary amino group as the active center. However, Stern & Melnick (141) found that thiamin, tested in Langenbeck's system, was completely inactive instead of being the most active of all the decarboxylating amines as could have been expected. The present explanation is that the primary amino group of thiamin possesses abnormal properties: it does not react with nitrite and can not be acetylated. This explanation suggests that the amino group exists only in the isomeric equilibrium:



The ultraviolet light absorption undergoes certain slight but characteristic changes on reduction of thiamin (142).

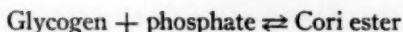
Thiamin can be phosphorylated to cocarboxylase in liver (143) and nucleated red blood cells (144, 145) and supposedly in many other organs as well. Kalckar (146) found that cell-free kidney extract contains a large amount of decarboxylase, but no purification experiments on the protein component have been carried out. Weil-Malherbe (147) has prepared a soluble yeast protein which was freed from cocarboxylase through acidifying in ammonium sulfate solution. The protein thus obtained could be reactivated by the addition of co-

carboxylase and subsequently contained the protein component of decarboxylase.

The chemical nature of the amino acid decarboxylases studied by Holtz and co-workers (148, 149, 150) are still unknown. They are probably not related to the thiamin proteids because of their inhibition by hydrogen cyanide.

ENZYME SYSTEMS THAT DECOMPOSE AND SYNTHESIZE GLYCOGEN

Through work carried out by Cori and co-workers and W. Kiessling, knowledge has been contributed to the important problem of the reversible synthesis and decomposition of glycogen. After Parnas, Ostern and co-workers showed that the Robison ester is formed from glycogen plus inorganic phosphate on the addition of muscle extract, Cori, Colowick & Cori (151) pointed out that glucose-1-phosphoric acid is formed as an intermediate product in the breakdown of glycogen. This is the so-called "Cori ester." The Cori ester is related to the Robison ester (glucose-6-phosphoric acid) through the influence of an isomerase or phosphoglucomatase. Since Davenport in 1926 had shown that amylase is only present in traces in the liver after the blood has been washed out, Cori & Cori (152) proposed the theory that glucose is formed in the liver through "phosphorolysis" and not through the activity of amylase. Kiessling (153, 154) obtained from yeast a protein fraction "C" that catalyzes the reversible reaction:



It was free from the isomerase that transforms the Cori ester to the Robison ester. Kiessling further found that protein C in turn can be split into one mainly phosphorylating portion and another mainly hydrolyzing portion through fractional precipitation with ammonium sulfate. It seemed remarkable enough that although the relation between the phosphorylating and hydrolyzing activities could be varied, the equilibrium in the reversible reaction remained constant with the value 5.2 for the ratio between inorganic phosphate and Cori ester, or the same value as found by Cori, Cori & Schmidt (155). The explanation is at present still unknown. The glycogen forms another phase as a colloid and does not take part in the equilibrium. Kiessling has even identified and isolated the reaction products of glycogen and Cori ester (154). Different authors had found that a co-ferment, supposedly adenylic acid, was necessary for the esterifi-

cation of glycogen. With sufficient amounts of protein C, added in Kiessling's experiment, adenylic acid, however, was not necessary. Protein C is not specific for glycogen, but for polysaccharides built up of glucose. With respect to proteins "A" and "B" of Warburg and co-workers, isolated from Lebedew juice, and Kiessling's protein C, the following should be noted: Meyerhof, Kiessling & Schulz (156) found that "A" catalyzes the interchange of phosphoric acid from phosphopyruvic acid to glucose. "A" and "C" work independently of each other. The "B" fraction contains isomerase which converts Cori ester to Robison ester. Thus, "B" and "C" added to glycogen and phosphoric acid give a reaction product which is Robison ester.

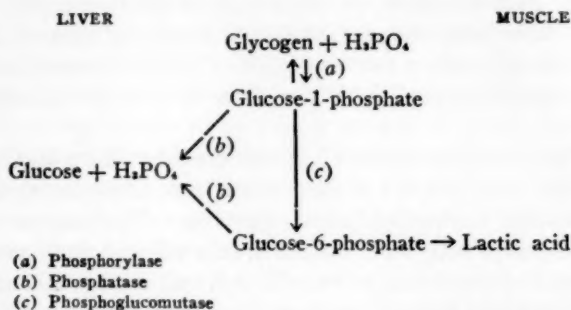
Cori and co-workers have studied the glycogen-enzyme system in different animal organs (155, 157, 158, 159). From dialyzed muscle extract, a phosphorylase and an isomerase could be separated through adsorption on aluminum hydroxide. The phosphorylase formed a polysaccharide in the presence of adenylic acid (but not inosinic acid or adenosine triphosphoric acid) which resembled glycogen in stability against hot sodium hydroxide, in hydrolysis with hydrochloric acid, and in insolubility in alcohol, etc., but which gave a blue rather than red-brown color with iodine. Only after long incubation could the red-brown color be obtained. If the phosphorylase was not completely free of isomerase the iodine reaction slowly disappeared again, thus giving further evidence of the reversibility of the reaction between glycogen, phosphoric acid, and Cori ester. Cori, Cori, & Schmidt (155) obtained a phosphorylase from liver which was free of isomerase and phosphatase and which formed a polysaccharide from Cori ester. This was identical with glycogen in all properties examined. The equilibrium constant between phosphate and glucose-1-phosphate was found to be 5.1 at 30°. Adenylic acid was necessary for the activity. Magnesium, manganese, and cobalt influenced the phosphorylating activity indirectly by stimulating the isomerase, through which the Cori ester that inhibits phosphorylation is changed over to Robison ester.

Cori, Cori & Schmidt have proposed the scheme shown on page 681 for the decomposition of glycogen.

According to Ostern and co-workers (160, 161), the phosphatase cleavage of the Cori and Robison esters to glucose and phosphoric acid is inhibited by fluoride.

Somewhat earlier than Kiessling, Schöffner (162) investigated the glycogenolytic enzyme system and came to similar but not as complete conclusions as the former. Gill & Lehmann (163), and Leh-

mann (164) have investigated the various inhibiting and accelerating activities of metal ions, -SH and -S-S- compounds on these enzyme systems. Euler & Bauer (165) have attempted purification of the phosphorylating starch-splitting enzymes in muscle tissue and sarcoma.



PHOSPHATASES

Numerous investigations of different phosphatases have been carried out during the past year. These show, in general, the same tendency to deal with the activation and inactivation of the phosphatases, their optimum pH and their substrate specificity. In the preparation of more highly purified products no progress has been made.

D. Albers (166) has shown that by prolonged dialysis of the alkaline kidney phosphatase a prosthetic group of low molecular weight splits off; it is not identical with magnesium ion. This confirms the earlier work of H. Albers and co-workers. The chemical nature of the prosthetic group is still entirely unknown. Massart & Dufait (167) found that previous results on the reactivation of the alkaline phosphatase of animals with manganese salts applies also to yeast phosphatase, whose optimum pH lies between 6 and 7. This reactivation is inhibited by fluoride. The greatest part of the activity disappears on dialysis. If reactivated with magnesium there is obtained a phosphatase which is inhibited by fluoride, but if reactivated with manganese there is no inhibition by fluoride. This difference is due to the fact that magnesium but not manganese forms inactive compounds with fluoride. Cloetens (168, 169) has pointed out the existence of two different β -glycerophosphatases in animal organs; the optimum pH of both lies around 9. The one, "I," requires magnesium ions in a concentration of 10^{-2} M to develop its full activity. It

is not inhibited by hydrogen cyanide but greatly so by fluoride. Phosphatase "II" is stimulated only to about twice its normal activity by magnesium and is inhibited almost completely by hydrogen cyanide but not by fluoride. Both I and II are activated by manganese. I is present abundantly in the liver, II in the kidneys. I can be isolated from II by inactivation of the latter in acid solution (170). The kinetics of I have been investigated and its structural formula proposed (171). Courtois and co-workers (172, 173, 174) have examined the substrate specificity and inhibiting factors of sweet almond phosphatase.

Engelhardt & Ljubimova (175) have extracted an adenosine triphosphatase from muscle tissue, which can free one molecule of phosphoric acid from adenosine triphosphate. The enzyme was precipitated together with and could not be separated from myosin. It was extremely thermolabile and could be denaturated in 10 min. at 37°. Strangely enough, it was stabilized by adenosine nucleotide in the same manner as the yellow ferment's free protein component is stabilized by flavinphosphate [Theorell (77)]. "Thus the primary energy yielding reaction in muscle, the hydrolysis of adenosine triphosphate, seems to be associated with the protein forming the anisotropic contractile part of the muscle fiber." Liebkecht (176) has found that adenosine triphosphate is split by bone phosphatase in the same way as by muscle phosphatase, so that the easily hydrolyzed phosphate is set free, after which the phosphoric acid at the C₅ carbon atom in the ribose is slowly released. This situation was to be expected in accordance with the formula of Lohmann for adenosine triphosphoric acid. The differing result of Barrenscheen & Iachimowitz (177) consequently could not be verified. The question as to whether the enzymes which dephosphorylate cozymase, adenylic acid, and inosinic acid are identical with the phosphatases which split glycerine phosphoric acids has been investigated by Das (178), who came to the conclusion that such is not the case. Differences between pH optima and heat stability were ascertained and a partial separation was possible. Roche & Bullinger (179, 180) have found that in the hemolysate of red blood cells an α -glycerophosphatase and a β -glycerophosphatase are present, which differ in pH optima and in their relations to magnesium, fluoride, and oxalate. In other experiments Roche and co-workers (181) demonstrated a close connection between phosphatase content and the degree of calcification in the scales and skins of different fish.

Several authors have studied blood phosphatases. Fujita (182)

advanced a quantitative method for the microdetermination of blood phosphatases. Iwatsuru and fellow workers (183, 184, 185) have pointed out a parallelism between the amount of blood phosphate and the number of myeloid leucocytes in myeloid leucemia and also in eosinophilia, dependent upon helminthiasis or the injection of *Ascaris* extract. Various dyestuffs injected in animals or mixed with blood or serum *in vitro* produced markedly less consistent results. Lundsteen & Vermehren (186) have proposed a method for determining serum phosphatase. In the blood plasma of a patient with prostatic cancer, there was shown to be a high content of "acid" phosphatase, supposedly identical with Kutscher's prostatic phosphatase.

Elvehjem *et al.* (187) determined the amount of phosphatase in blood and bone in chick perosis. The phosphatase content decreased and the decrease could be shown even before the symptoms of perosis appeared. Therefore, the low phosphatase content can possibly be etiological to the disease which appears with a diet high in calcium and phosphate, but poor in manganese. Addition of manganese salts to the diet produces a normal phosphatase content and prevents perosis. Normal and rachitic rats show no difference in the phosphatase content of the organs (188).

The velocity of phosphate cleavage has been determined for a number of phosphoric acid esters (189). The activation energy for bone phosphatase at the optimum pH with β -glycerophosphate as the substrate is $9,940 \pm 140$ cal. per mol calculated according to Arrhenius' law (190). An alkaline phosphatase from brain has its isoelectric point at pH 8.8 to 9 according to electrophoretic experiments; this coincides with the point of optimum activity (191). Galactose-1-phosphoric acid is possibly a normal metabolite in galactose metabolism in yeast and liver (192). The phosphoric acid ester is concentrated in the liver during galactose assimilation.

Kalckar (193, 194) pointed out earlier a connection between the phosphorylating and oxidizing processes in kidney extract. He shows now that all hydrogen donators that stimulate respiration (alanine, glutamic acid, citric acid, malic acid, fumaric acid) also stimulate phosphorylation. A whole series of different phosphorylation products, formed in these processes, has also been demonstrated. In this manner, adenylic pyrophosphate is formed from adenylic acid; fructose diphosphate and dihydroxy acetone phosphate arise from glucose; fructose, fructose-6-phosphate, and 1- α -glycerophosphate from glyc-
erine.

UREASE

Sizer (195) has investigated the temperature activation of the urease-urea system. The activation energy was found to be either 11,700 or 8,700 cal. per mol. As known before, the decomposition of urea to ammonia and carbamic acid is reversible. Oxidizing agents seem to promote the synthesis, while reducing agents promote the decomposition (196). This is analogous to the results of Itoh *et al.* (213) on lipase.

CARBOHYDRASES

This subject has been rather fully treated by Myrbäck in the *Annual Review of Biochemistry* for 1939, so that a short review of the work carried out during 1939 will be sufficient.

Leibowitz & Hestrin (197) show that takamaltase and takasucrease may be separated preparatively, which thus supports the Leibowitz theory in opposition to that of Weidenhagen.

Helferich and co-workers (198, 199, 200, 201) have continued their investigations of the β -glucosidases in sweet almond emulsin and other related problems. These enzymes, in common with all the various carbohydrases, seem to be far from pure. Fishman (202, 203) has continued a study of the β -glucuronosidases from spleen.

Zechmeister and co-workers (204, 205) have succeeded through chromatography in separating the active enzymes from emulsin and from extracts of *Helix pomatia* into chitinases and chitobiasis, of which the former act upon chitin and chitodextrin, the latter upon the cleavage products of lower molecular weight. Neuberger & Pitt Rivers (206) have similarly identified through chromatography a β -glucosidase and a β -glucosaminidase fraction from a glycerin extract of *Helix pomatia*. The authors conclude from their experiments that chitobiose, chitotriose, and chitin all have the β -configuration. Aizawa (207) has used *p*-nitrophenol- α and *p*-nitrophenol- β glucosides as substrates in his comparisons of the activity of emulsin and takadiastase. The use of these substrates has the advantage that the rapidity of the cleavage can easily be determined by the color of the freed nitrophenol. Emulsin only splits the β -glucoside while the takadiastase splits both. The α -glucoside is split by all the investigated organ extracts from rabbits, and the β -glucoside only by extracts from the liver and kidneys. The β -galactoside has also been investigated in a corresponding manner (208). Stoll & Renz (209) examined the enzymes that cleave the heart glucosides in *Strophanthus* seed.

Myrbäck & Örtenblad (210) find that the β -amylase in barley is bound to an insoluble protein and is inactive in this form. It is released in active form by proteolytic enzymes. The statements of Chrzaszcz and co-workers concerning the activation of amylase by hydrogen sulfide are interpreted by Myrbäck, who claims that the Polish author's amylase was inactivated by copper impurities which could be removed by hydrogen sulfide, thus producing the active enzyme. This view is supported by Janicki (211), who maintains, however, that ascorbic acid also plays an important role in this connection.

VARIOUS HYDROLYZING ENZYMES

Kunitz (212) has crystallized a ribonuclease from beef pancreas which splits yeast nucleic acid into dialyzable products. Phosphoric acid is not set free. Itoh *et al.* (213) find that the splitting action of serum lipase and milk esterase is weakened by oxidizing agents and strengthened by reducing agents. Klein (214) has purified somewhat a cholesterol esterase from pancreas.

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